

# **Epidemiology of Rift Valley Fever In Sudan**

**By**

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## ***Preface***

*This work was conducted in the Department  
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and Professor Imad Eldein ElAmin Aradaib.*

***Dedication***

*To the soul and memory of my parents*

*To the soul and memory of my dear*

*brother*

*ElHassan and dear sister Buthiana*

*To My dear husband for his ultimate*

*Patience, Support and encouragement*

*To my family with great love...*

*To all who made this work possible?*

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## **ABSTRACT**

This study was designed to evaluate the present situation of RVF among animal population in different regions of Sudan. The study included serosurveillance and diagnosis of suspected RVF cases. Two serological tests were used to detect anti-RVFFV antibodies: Enzyme linked immunosorbent assay (ELISA) and Indirect fluorescent antibody test (IFA).

Sera were collected and tested in the year 2001 a total of 1351 (491 bovine, 423 ovine and 437 caprine). Using Sandwich ELISA for the detection of anti-RVFFV IgG antibody, the overall prevalence rate was ranging from 3.4% to 10% in animals from Buram (Western Sudan) and Shandi (Northern Sudan) respectively. All positive reactions were detected in sheep (15 samples) and goats (1 sample). 1543 serum samples (612 bovine, 313 caprine and 618 ovine) were tested by Capture IgM ELISA during 2001. No anti-RVFFV IgM antibody was detected in all these tested sera.

In August 2002 a febrile disease spreaded among cattle in every locality in Khartoum and River Nile States manifested by high abortion rates reaching as high as 70% and deaths of aborted foeti. This raised the suspicion of RVF outbreak. Capture IgM ELISA test has been developed with an inactivated viral antigen. A total of 107 bovine serum samples collected from Khartoum and River Nile State as follows: 55 samples from East Nile Province, 35 samples from Alshajara and 17 samples from the River Nile State, were tested for anti-RVF IgM antibodies using Capture IgM ELISA.

Thirty one out of these samples were found positive. The percentage positivity was 25.5%, 14.2% 70.5% respectively.

In the year 2003 no clinical disease was reported. Moreover all tested positive in the year 2002 were culled or removed from the herds. In the year 2005, about 410 bovine serum samples from El damazine (60), Elgadarif (31), Kassala (269) and El showak (50) and 257 ovine samples from Kosti and Elshowak were tested for anti-RVF IgM antibodies using IgM ELISA. The percentage positive was 6.6% and 1.8% in Eldamazine and Kassala respectively. No anti-RVFPV IgM antibody was detected in the ovine sera.

In this study Indirect fluorescent antibody test (IFA) was used for serological diagnosis of RVF using inactivated antigen, 100 bovine serum samples were tested for anti-RVF antibodies, 23 out of these were positive. The degree of agreement between ELISA and IFA tests were very high i.e. IFA test can be used in the diagnosis of RVF.

For early and rapid detection of RVF virus and an efficient surveillance system of a single tube reverse transcriptase polymerase chain reaction (RT-PCR) method focusing on the NSs coding region of the S segment was developed and used RVF virus genome, resulting in the synthesis of 363 bp DNA amplifiers were detected in RNAs extracted from RVFPV vaccine strain (Smithburn) and tissues (spleen and liver) from aborted feti. The assay was specific for RVFPV and did not amplify any other hemorrhagic virus. When serial dilutions of RVFPV were mixed with DDW, the minimal

detection limit was 0.5 plaque forming units. The RT-PCR was efficient for the detection of RVFV RNA.

Entomological surveillance data about mosquitoes in Khartoum was recorded during the period of the study.

# الخلاصة

2001 2005 .

0

. ( ELISA)

Indirect fluorescent test( IFA test )

.antibody

( 437 423 491) 1351

%3.4 IgG (ELISA)

%10

.2001 IgM .

2002

31)

(%70 ) (

107

, ,

. %70 %14.2 ,%25.5

2003

. 2002

410 2005

%1.8 %6.6

257 .



( Smithburn )

.( Vero cells )

( RT-PCR )

# **Epidemiology of Rift Valley Fever in the Sudan**

## **INTRODUCTION**

Rift Valley fever (RVF) is a peracute or acute zoonotic disease of ruminants in Africa. It is caused by a single serotype of a mosquito-borne Bunya virus of the genus Phlebovirus, which consists of at least 51 virus serotypes. The disease occurs in climatic conditions favoring the breeding of mosquito vectors and is characterized by liver damage. The disease is most severe in sheep, goats and cattle, in which it produces abortions in pregnant animals and a high mortality rate in the newborns. Older non pregnant animals, although susceptible to infection, are more resistant to clinical disease. There is a considerable variation in the susceptibility to RVF in different animal species. Those breeds or strains that are exotic to Africa or from areas where RVF is not endemic tend to be more susceptible. Camels suffer an inapparent infection but abortion rates can be as high as in cattle (OIE, 2004).

Humans are susceptible to infection by handling infected materials and through transmission by mosquito vectors. Infection of humans by vectors is a striking feature in Countries with relatively small population of animal hosts. In such areas, RVF may be recognized first in humans. It has caused a serious disease in laboratory workers and must be handled with high level biosecurity (OIE, 2004).

The virus caused massive epidemics in animal populations across Africa (Eisa *et al*, 1977). Modifications in the ecology, biology of the virus and environmental conditions appeared to be responsible for the resurgence and emergence of the virus, (Meegan and Bailey. 1989; Peters *et al*.1994). RVFV has been isolated from cattle in Aswan South Egypt in 1977(Abdelwahab *et al.*, 1978) a recent epidemic of the disease was once again reported in 1997 the virus was recovered from cattle at the University of Assiut, (Abdelhakeem *et al.*, 1998). This virus was described for the first time in Saudi Arabia in the year 2000 (FAO, 2000). The virus was also recovered from clinically normal, naturally infected horse. Also the economic losses attributed to the often-sub clinical disease are difficult to estimate, (FAO, 2003).

This emerging pathogen constitutes a major hazard to many African and Asian countries including the Sudan, Egypt, Somalia, Mauritania, Saudi Arabia and Yemen, (FAO, 2003).

**Objectives: -**

1. To conduct cross-sectional serosurveillance of RVF virus antibodies in Sudan,
2. To apply IgM ELISA for diagnosis of RVF.
3. To conduct serological survey using IgG and IgM ELISA detection technique.
4. Establishment of Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) technique for diagnosis of RVF.
5. Collection of epidemiological data on the disease and its vectors.

The information gained will be used to construct effective control measures that could be implemented in the case of outbreaks or for animal trade purposes.

## CHAPTER 1

### 1. LITRATURE REVIEW

#### 1.1. Causative agent:-

Rift valley fever virus (RVFV), is a member of the phlebovirus genus in the family Bunyaviridae (McIntosh *et al.*, 1980), mature particles, liberated by the disintegration of Vero cells, contained ribosome-like structures within the nucleocapsid, which is surrounded by a typical unit membrane through which are inserted 350-375 surface spikes whose inner ends were incorporated into the nucleocapsid structure (Ellis *et al.*, 1988). In the negatively stained material, the overall diameter of the virion was 90-110 nm; the spikes were 10-18 nm in length and 5 nm in diameter (Ellis *et al.*, 1988). The viral particles are spherical or pleomorphic, depending on the method used for fixation as with all members of the family Bunyviridae (Gonzales-Scarano and Nathanson, 1996 ). No significant antigenic differences have been detected between RVF isolates.

The virus is very stable in serum and at temperature lower than  $-60\text{ }^{\circ}\text{C}$  or after freeze-drying, and in aerosols at  $23\text{ }^{\circ}\text{C}$  and 50-85% relative humidity. It is inactivated by lipid solvents ,formalin and pH below 7, The virus grows readily in all continuous cell lines and embryonated chicken eggs as well as in a variety of laboratory animals (Seifert, 1996 ).

### **1.1.1. Genome of Rift Valley Fever Virus (RVFV):**

Like all the phleboviruses, RVF virus possesses a tripartite genome consisting of negative sense single-strand RNA segments designated L (large), M (medium) and S (small). The L segment codes for the L viral polymerase. The M segment codes for the precursor to the envelope glycoproteins G1 and G2 and the non structural proteins 14 and 78 kDa. The S segment codes for the nucleocapsid protein N and the non structural protein sequences (NSs) in an ambisense strategy (Vialat *et al.*, 1997)

#### **1.1.1.1. S segment (Website 1):**

**GenBank Accession Number:** NC-002045. **Size:** 1690.

**Gene Count:** 2 genes. The S segment codes for the nucleocapsid protein N and the non structural protein NSs in an ambisense strategy (Vialat *et al.*, 1997).

The gene organization and expression of the S segment are the major features that distinguish the Phlebovirus genus from Bunya viruses, Hantaviruses, and Nairoviruses, but are similar to those Topoviruses (Giorgi, 1996). The sequences and coding strategies of the S RNAs of two viruses, Toscana (TOS) and the M12 derivative of Rift Valley fever ZH-548 (RVF, Phlebovirus genus, Bunyaviridae) have been determined from cDNA clones and compared to the previously published sequences of Punta Toro (PT), Sand fly fever Sicilian (SFS), and Uukuniemi (UUK) viruses. All five viruses exhibit an ambisense coding strategy for their small (S) RNA species, i.e., one gene product (the NSs protein) is encoded in

the 5' half of the viral RNA, a second (the N protein) is encoded in the sequence complementary to the 3' half,(Giorgi *et al.*, 1991).

#### **1.1.1.2. M Segment (Website 2):**

**Gene Bank Accession Number:** NC\_002044 M25276 M11157

**Size:** 3884 or 3885 bp (Website 2).**Gene Count:** 2 genes.

The M segment codes for the precursor to the envelope glycoproteins G1 and G2 and the non structural proteins 14 and 78 kDa (Vialat *et al.*, 1997). The sequence data show that all viruses possess a single large ORF extending the length of the M segment in the viral complementary RNA. This ORF codes for a polypeptide precursor of the viral glycoproteins G1 and G2 (Giorgi, 1996).

#### **1.1.1.3. L Segment (Website 3):**

**Gene Bank Accession Number:** NC-002043. **Size:** 6606 (Website 13) **Gene Count:** 1 gene.

The L segment codes for the L viral polymerase (Vialat *et al.*, 1997).

### **1.2. History and World distribution:-**

RVF virus primarily infects domestic cattle causing massive epidemics in human populations across Africa (Eisa *et al.*, 1977).

Recently RVF enter Saudi Arabia and Yemen in September 2000 (Jup.*et al.*, 2002)). Modifications in the ecology, biology of the virus and environmental conditions appear to be responsible for the emergence of the virus (Meegan and Bailey., 1989; Peters *et al.*, 1994).Tara (2002) stated that outbreak of RVF is often associated with periods of heavy rainfall and construction of dams, after which the mosquito population flourishes.

RVF was first identified as an outbreak in exotic wool producing sheep and illness in humans that occurred in the Rift Valley of Kenya after heavy rainfall in 1930-31,(FAO,2003). Outbreaks have since occurred in the highlands of Kenya at irregular intervals of 3-15 years. The most recent epizootic in the East African region was in 1997-98 in the drier areas of northeast Kenya and southwest Somalia after heavy El Niño-associated rains. This caused human deaths and some livestock losses, particularly of camels, but more significantly, disruption to livestock exports to the Middle East from the Horn of Africa (FAO, 2003). The disease was first recorded in the Republic of South Africa in 1950, when a major epizootic in South Africa caused an estimated 100 000 deaths and 500 000 abortions in sheep. A second extensive epizootic occurred in Namibia and South Africa in 1974-75. Periodic severe outbreaks have also been experienced in Mozambique, Zambia and Zimbabwe (FAO, 2003). In 1987 RVF was reported in Mauritania following the building of the Diama Dam at the mouth of the Senegal River. This is an area where the disease is endemic, but had often existed as only an inapparent infection. Never before had RVF been seen with such a high morbidity and mortality (Lederberg *et al.*, 1992), a further outbreak of the disease occurred there in 1998.

In 1973, RVF outbreaks occurred in irrigated areas of the Sudan. (FAO, 2003). In 1977, the virus was isolated from infected cattle during an epizootic of the disease in Aswan, South Egypt (Abdelwahab *et al.*, 1978; Arthur *et al.*, 1993). The disease was recognized there and caused an estimated 600 human deaths as well



as heavy losses in sheep, goats, cattle, buffaloes and camels along the Nile Valley and Delta. RVF outbreaks again occurred in Egypt in 1993. (FAO, 2003). A recent epidemic of the disease was, once again, reported in Aswan, Egypt in the summer of 1997, the virus was isolated from calves, ((Abdelhakeem *et al.*, 1998).

RVF virus is probably present in all countries of sub-Saharan Africa. Many of these countries, outside eastern and southern Africa, do not have populations of the highly susceptible exotic livestock breeds that act as indicators of RVF virus activity. Human disease may be the first indication that RVF virus amplification is occurring at high levels in these countries, where the indigenous ruminants may not show any clinical signs of disease other than insignificant low levels of abortion, (FAO. 2003).

Until recently RVF was thought to be restricted to Africa. However, it was reported in the Tehama area of both Saudi Arabia and Yemen in September 2000. The Tehama plain - about 50 km wide - is in the west of these countries, between the mountains and the Red Sea, on the eastern side of the Great Rift Valley. It is a semi-arid zone with alluvial fanning from the mountains that form the scarp of the Rift. Its ecological characteristics are similar to those in the corresponding western side of the Rift Valley in Africa, where RVF occurs. RVF virus activity is highly associated with such limited riverine alluvium zones. There were extensive abortions in sheep and goats and some 855 severe human cases with 118 deaths. The virus was similar to that circulating in Kenya and Somalia in 1997-98. (FAO, 2003). RVF however, has the potential

for further international spread, particularly with the climatic changes that might be expected with global warming. High-risk receptive areas are, for example, the Tigris/Euphrates Delta zone to the northeast in Iraq and the Islamic Republic of Iran, (FAO, 2003)

It is worth mentioning that, RVFV was thought to be diagnosed in 1987 based on clinical signs and histopathological findings in the liver of sheep in a farm in the Ankara region and in cattle in a farm in southwest Turkey, (Girigin, and Akosoye, 1988).but was never virologically or serologically confirmed

In the Sudan, the first survey for antibodies to RVFV in human populations was conducted in the Southern region. The prevalence of the infection was found to be 6.7% as determined by agar gel diffusion test, (Findlay *et al.*, 1936).

An epizootic of the disease was also reported in Kosti District, the White Nile Province of the Sudan in 1973, it covered almost every single locality in the District and spread northwards to Blue Nile Province and northern part of the White Nile Province. Sheep, goats, cattle and humans were involved. (Eisa *et al.*, 1977).Mortality rates was highest in lambs (96%) followed by goats kids and calves (Eisa *et al.*, 1977). Subsequently, the virus was isolated and identified as RVFV using serum neutralization test (SNT). Hyperimmune rabbit antiserum to the Egyptian strain (Zagazig) of RVFV was employed in the SNT (Eisa, and Obeid, 1977; Eisa *et al.*, 1977). In a later study, Eisa (1984) reported a preliminary survey of domestic animals of the Sudan for precipitating IgG antibodies to RVFV antigen. The prevalence of

the infection was 34.3% in sheep, 33.2% in cattle, 22% in goats, 7.9% in camels and 4% in donkeys. Antibodies to RVFV were not detected in sera from horses. Therefore, it was suggested that horses are unimportant host in the epidemiology of the disease (Eisa, 1984). However, the virus was recovered from, clinically normal, naturally infected horse and hence the level of virus infection in this animal species has probably not been estimated sufficiently (Eisa, 1984). A recent serological survey was conducted in patients admitted to Hag Elsafi Hospital, Khartoum North, Sudan, for detection of antibodies to RVFV. Approximately, 3% of the patients were recently infected with RVFV, as determined by detection of IgM antibodies (Kambal, 1997).

### **1.3. Epidemiology:-**

#### **1.3.1. Transmission:-**

RVFV is an arthropod-borne virus. The virus is transmitted by mosquitoes *Aedes* or *Culex*, However, other arthropods, such as *Culicoides imicola*, and ticks may also serve as mechanical vectors for RVFV (Eisa *et al.*, 1977). The importance of these vectors and their distribution are directly related to the distribution of RVFV strains. In Central Sudan, the prevalence of the virus antibodies showed a marked seasonal pattern, with the infection level being higher in the rainy months which coincided with a high population density of the invertebrate vectors (Eisa, *et al.*, 1980). Biological fluids, including semen and milk, may serve as a source of infection or play a role in the transmission of the disease during viremia (FAO, 2003). However, additional research is necessary to confirm this assumption. Based on

these surveys, it was concluded that RVFV circulates across the Sudan in a south-north range along the River Nile Valley with little or no extension to the dry areas on the east and west (Eisa *et al.*, 1980), the dissemination out side the African continent to Saudi Arabia. It is most probable that the RVFV crossed the Red Sea via the crossing winds and thereby reaching the Arabian Peninsula in Asia. It is unlikely also that the virus was introduced in Asia by importation of infected animals from the African continent. If the viral hemorrhagic fever reported in Turkey (Girigin and Akosoye, 1988) among sheep and cattle were really RVF, the virus would have been carried by wind in the same manner. The virus will probably spread to Europe in the future. Bluetongue virus (BTV), an arthropod-borne virus of the genus orbivirus in the family Reoviridae (Borden, *et al.*, 1971), might have been introduced by wind blown arthropods to Tunisia (Africa) and Italy (Europe) simultaneously. These are different geographical locations where BTV has never been reported before (OIE, 2004).

### **1.3.2. Non-vector transmission of RVFV:-**

Unlike, human, non-vector transmission of RVF virus is not considered to be important in livestock. The risk of human-to-human infection through direct contact appears also to be very low. However, in addition to mosquito transmission, humans are easily infected by contact with the body fluids of infected animals through contact with abraded skin, wounds or mucous membranes or by inhalation of aerosols. Thus, the slaughter of infected animals, necropsy procedures and laboratory handling of tissues and isolated viruses are activities carrying a high risk of disease transmission. (FAO, 2003).

RVF outbreaks have not been seen in urban consumer populations suggesting that the fall in pH of meat with maturation inactivate the virus.

Low concentration of the RVF virus is found in the milk of infected animals and the connection has been made between human infection and the consumption of raw milk. Clearly this could be a significant factor in pastoral communities where raw milk is a major component of the diet. (FAO, 2003).

It is suspected, and it is highly likely given the high concentration of RVF virus in the blood of affected animals, that infection can be transferred between animals when they are vaccinated or blood sampled in succession with the same needle during an epizootic (FAO, 2003). Outbreak of RVF occurs generally when particularly heavy, prolong and, often, unseasonal rainfall favors the breeding of mosquito vectors. Epidemics in most of eastern and southern Africa occur in 5 to 20 year cycles, but, in the dry semiarid zones of eastern Africa the periodicity is 15 to 30 year, (FAO report, 2003).

### **1.3.3. The Environmental Factors:-**

The environmental conditions appear to play a crucial role in the distribution of the arboviruses in any continent, where the activity of the vector is likely to be highest during late summer and early fall. In contrast, the activity of the vector is usually suppressed during the winter season. However, transmission of RVFV during the winter season is not uncommon (Peretorius *et al.*, 1997). The overwintering mechanism for viral transmission is yet to be explained. Cyclonic

winds and warm temperatures determine how far the infection would be transmitted. The vectors can be carried with the wind for hundreds of miles, thus transmitting the infection to different continents, (Peretorius *et al.*, 1997).

#### **1.3.4. Reservoir Hosts:-**

Since the duration of viremia was not determined in each specific ruminant species, it would be extremely difficult to predict which animal species is likely to be the reservoir host of the disease. Camels may contract the infection during epizootic of RVFV. However, the infection is usually subclinical and abortion is the only sign of the disease in pregnant she-camel (Eisa *et al.*, 1977). Subclinically infected camels and other wild animals could probably serve as reservoir hosts for RVFV if viremia persists for a long time in these particular animal species. Very little information is available on the impact of RVFV in wild animals. In addition, rodents were suspected as a potential reservoir of RVFV in South Africa (Peretorius *et al.*, 1997).

Nothing is known about the susceptibility of avian species to RVFV; but this situation may parallel that of Crimean-Congo hemorrhagic fever (CCHF). In South Africa, workers in Ostriches farm acquired CCHF when handling tick infested slaughtered ostriches; the ostriches may serve as amplifying host for the virus without showing clinical signs of the disease. Thus providing virus for insect transmission to more susceptible animal populations. (Swanepool, and Coetzer, 1994). Nevertheless, the exact reservoir hosts of RVF virus in different continents are yet to be determined.

### **1.3.5. Populations at Risk:-**

Studies have shown that sleeping outdoors at night in geographical regions where outbreaks occur could be a risk factor for exposure to mosquito and other insect vectors. Animal herdsman, abattoir workers, and other individuals who work with animals in RVF-endemic areas (areas where the virus is present) have an increased risk for infection. International travelers increase their chances of getting the disease when they visit RVF-endemic locations during periods when sporadic cases or epidemics are occurring, (FAO, 2004)

### **1.3.6. Inter-epidemic Virus Survival:-**

During the long interepidemic periods, low levels of virus activity may occur in certain foci within the epidemic and enzootic areas and these will remain undetected unless intensive surveillance activities are carried out. Virus activity may be revealed by random isolations from mosquitoes or by occasional human disease. Small local RVF outbreaks may occur, when and where the micro-environmental conditions are favourable and susceptible livestock are present. However, the incidence of infection is usually so low to be detectable. Clinical disease in humans or animals is generally missed in the absence of specific, well-focused active surveillance. Transovarian transmission of RVF virus occurs in some species of *Aedes* mosquitoes of the Neomelaniconium group, (Linthicum *et al* 1985). The eggs of these mosquitoes, and the virus that they carry, may remain viable for very long periods in the mud of dried-up surface pools or shallow depressions (locally known as *dambos* or

pans), or in floodplains. Infected mosquitoes hatch from these when they are again flooded. This is the reason why the virus persists during prolonged interepidemic periods in the grasslands and semi-arid regions of Eastern, Western and Southern Africa, (FAO, 2003).

#### **1.3.7. Cryptic or (Sylvatic) RVF:-**

In Africa, the infection cycle among indigenous, domestic and wild vertebrate animals and mosquitoes is subclinical, both in livestock and people. In the rain forest and wetter wooded areas of the country, the virus circulates silently between wild and domestic species and insect vectors. This is referred to as cryptic or sylvatic RVF virus activity. Cryptic RVF is extremely difficult to identify and occurs in most of the countries of sub-Saharan Africa, (FAO, 2003).

#### **1.3.8. The generation of epidemics and inter-epidemic persistence of RVF infection:-**

Recurrent viral activity occurs in localized areas in southern and eastern Africa where transmission of RVF virus to ruminants occurs during most years. This provides one of the keys to understand virus survival during inter-epidemic periods. The other key to virus persistence lies in the biology of certain floodwater-breeding Aedine mosquitoes. These aedine mosquitoes endure dry periods as eggs which can survive for long periods, possibly several seasons, in dried mud. It is obligatory for these eggs to be subjected to periods of drying before they can hatch. RVFV is transmitted transovarially in certain Aedes species and infection persists for their life. Ideal conditions for the breeding of aedine and other



mosquitoes species are found in low-lying shallow depressions, termed dambos, which flood when abnormally heavy rainfall raises the water table sufficiently. An explosive increase in the *aedine* mosquito vector populations follows with increased transmission of RVF virus mainly to cattle, on which they feed selectively. Amplification of the virus in cattle provides infection for a range of secondary or epidemic mosquito vectors, such as *culicine* and *anopheline* mosquitoes, which transmit infection to many susceptible ruminant species and humans. (FAO, 2003).

The generation of epidemics seems generally to be associated with the simultaneous intensification of vector and virus activity over large areas within which the virus is already present in some sites, rather than lateral spreads from cryptic endemic foci. After prolonged draughts during which dambos remain dry for many years, it is possible that *aedine* mosquito populations could decline to a point where they and RVF virus disappear from large areas. Considerable virus amplification would then have to occur before the disease again becomes evident or it would need to be reintroduced through long range dispersal of viraemic animals or infected vectors (FAO, 2003).

Flooding and humid weather conditions favour the breeding not only of mosquitoes but also of the other biting insects which are potential and mechanical transmitters of RVF virus. Eggs of species which breed in water, other than those of *aedine* mosquitoes, cannot survive dry conditions and these recolonise flooded dambos from nearby rivers or dams. Successions of vector species become

available once flooding occurs. Mechanical transmission of infection by mosquitoes, midges, phlebotomids, stomoxids, simuliids and other biting flies appears to play a significant role in epidemics. It has been suggested that *Hyalomma* species ticks on cattle could have spread RVF infection to West Africa and Egypt but this is now considered unlikely. Ticks appear to play no role in the epidemiology of RVF. (FAO, 2003).

#### **1.3.9. Predictive Epidemiology:-**

Early work in Kenya showed a close association of epizootic RVFV activity with periods of prolonged and heavy rainfall. The climate in much of Africa depends upon the characteristics of the inter-tropical convergence zone (ITCZ). This is the area of convergence of cold air currents from the north of the continent with warm air from the south. Its density and magnitude determine the levels of cloud cover and precipitation, (FAO, 2003).

Initially, prediction of RVF virus activity was made on the basis of the occurrence of 2-5 times the mean annual rainfall, which caused flooding of grassland water pans or dambo formations. It was noticed that such flooded habitat, allowed huge mosquito populations to develop at periods when clinical RVF occurred. Statistical information was derived from the actual rainfall figures, the number of rain days/month and the rolling mean, which correlated well with epizootics of RVF over 25 years. (FAO report, 2003).

The advent of Remote Sensing Satellite Imagery (RSSI) derived from Landsat, SPOT and later Synthetic Aperture Radar

allowed the use of more sophisticated tools to predict RVF epizootic activity over much wider areas. Ground truth data was generated to correlate with changes in the Cold Cloud Density (CCD) and the Normalized Differentiated Vegetation Index (NDVI). This is a measure of the intensity of green vegetation. The NDVI index could then be correlated with the conditions, which allowed the emergence of the *Aedes* mosquito vectors of RVF. Pilot studies with the NDVI values in other parts of Africa have allowed Risk Assessments for epizootic RVF to be made, by calculating the difference between the normal, expected and the actual rainfall. The initial results appear to be promising and may be applicable on a much wider basis to similar biotopes in other parts of Africa. Ocean temperature changes have now been shown to be correlated with the RSSI data and provide a further tool for the prediction of epizootic RVF activity. Climatic changes usually occur on a sub-regional basis rather than continental (FAO, 2003).

#### *1.4. Clinical Signs:-*

The most prominent clinical manifestation in all animal species involved was severe icterus and abortion according to field observations. The duration of the disease is very short extending from 2 to 7 days, after which the animals succumbed or recovered (Eisa *et al.*, 1980).

##### **1.4.1. Sheep and goats:-**

Clinical disease occurs in susceptible sheep (such as imported wool sheep) of all ages, but is most severe in young lambs. The morbidity rate in infected flocks approaches 100%. The mortality

rate may be as high as 95% in lambs less than one week old, about 40-60% in weaner lambs, and 5-30% in adult sheep. The abortion rate may approach 100 %.( OIE, 2004).

In peracute cases, sheep are either found dead or suddenly weaken and collapse when driven. In acute cases, there is a very short incubation period - less than 24 hours - followed by pyrexia, rapid pulse, weakness, unsteady gait, vomiting, mucopurulent nasal discharge and death in 24-72 hours. Other signs often observed are lymphadenitis, colic, hemorrhagic diarrhea and petechial or ecchymotic hemorrhages in visible mucous membranes. (OIE, 2004).

Subacute disease is more likely in adult sheep. Diphasic pyrexia is accompanied by anorexia and weakness. There may be some vomiting and evidence of abdominal pain, with or without hemorrhagic gastroenteritis. Hepatitis with jaundice develops in most cases. Abortion is an almost inevitable consequence of infection of pregnant ewes, and may occur in either the acute or convalescent stages of the disease (OIE, 2004).

RVF in goats is similar to that in sheep but is usually not quite so severe. It is important to remember that the indigenous hair sheep and goats in Africa may show none of the above signs and no clinical signs other than some abortions. Flocks with or adjacent to exotic animals with severe RVF disease may show no signs (FAO, 2003).

#### **1.4.2. Cattle and water buffaloes:-**

In cattle, as in sheep, the most severe disease is seen in young animals. The mortality rate in exotic calves of *Bos taurus* breeds, such as Friesians, may be up to 30 percent, or even higher in neonates. Some animals up to 6 and even 12 months may be severely ill and debilitated with hepatitis and jaundice for some months. The acute disease is similar to that in sheep. In adult cattle the mortality rate is less than 2-5% while pregnant cows abort. They may show pyrexia, a sharp drop in milk production, lymphadenitis, anorexia and malaise. Hemorrhage from the mouth and nares often occur, with colic and hemorrhagic diarrhea. In extensively ranched cattle, abortions may not be observed and a drop in calving rates may be the only sign recognize (OIE, 2004).

#### **1.4.3. Camels:-**

Although infection is generally subclinical in mature animals, pregnant she-camels may abort at any stage of pregnancy and neonatal deaths can occur. Abortion rates of 70 % of those pregnant animals occurred with many deaths in foals 3-4 months of age (Eisa *et al.*, 1977).

#### **1.4.4. Humans:-**

After an incubation period of two to six days, patients experience an influenza-like disease with a sudden onset of fever, debility, headache, backache and other muscle pains, and often photophobia and vomiting. The fever is diphasic. There is usually a degree of liver damage with jaundice. In uncomplicated cases, the illness generally resolves itself within a week. Many cases are mild.

However, RVF in people who have pre-existing diseases such as schistosomiasis or malnutrition may be severe or even fatal. Complications of RVF that occur in a small percentage of human infections include, (FAO 2003):

- . Hemorrhagic fever with hepatitis, which is often fatal
- Meningo-encephalitis.
- Retinitis, with permanent loss of vision in 1-10% of such cases

#### 1.5. Pathology:-

RVFV infected mosquitoes or insect vector transmits the infection by biting. The virus invades the subcutis and join the lymphatic and eventually reaches the regional lymph nodes where initial replication of the virus takes place, the virus joins the blood stream causing viremia. Subsequent replication takes place in the reticuloendothelial system. The persistence of viremia for along time in cattle could probably be explained by the association of the virus with blood cells (Aradaib *et al*, 1997). In addition, it is probably that the virus hides in the liver within the hepatocytes for such a long time. The liver is the most seriously affected organ and hence the name (enzootic hepatitis), which was, suggested when the virus was described for the first time (Daubney, 1931). This is due to destruction of the hepatocytes by rapid replication of the virus in this vital organ leading to necrotic hepatitis.

### **1.5.1. Gross pathology:-**

At necropsy the most characteristic lesions in all animal species are various degrees of necrosis of the liver and extensive jaundice of whole carcasses (Eisa *et al.*, 1980). There are also petechial and ecchymotic hemorrhages on all serous surfaces, lymph nodes, subcutis and the kidneys and in various tissues. Necropsy findings of the peracute cases include necrotic hepatitis particularly in young animals, hemorrhagic diarrhea and yellow gelatinous subcutaneous and intramuscular edema, pulmonary edema, straw-colored transudate in the thoracic pericardium and abdominal cavities. Hemorrhagic enteritis is associated with bloody diarrhea, (Abdelhakeem *et al*, 1998). When severely affected for example, in young lambs the liver is swollen and the capsule becomes tense, giving an external impression of firmness. However, on section the organ is quite friable, congested and contains many hemorrhages. When not masked by blood, the color of the liver ranges from pale grey-brown to yellow-brown. Numerous grey-white foci, 1-2 mm in diameter, are scattered throughout the parenchyma. The gall bladder may be edematous and contain petechial or ecchymotic hemorrhages. All lymph nodes are likely to be enlarged, edematous and hemorrhagic. (FAO, 2003)

The gastrointestinal tract exhibits varying degrees of inflammation, from catarrhal to hemorrhagic and necrotic. Petechial or ecchymotic hemorrhages are present in most internal organs. Ascite, hydropericardium, hydrothorax and pulmonary edema may

be present. The fluid in the body cavities is frequently blood stained and the carcass is icteric, (FAO, 2003).

#### **1.5.2. Histopathology:-**

In the livers of young animals, there are well-defined primary foci of severe coagulative necrosis, which may be centrilobular. These are accompanied by diffuse and massive pan-necrosis involving most (or all) of the rest of the parenchyma. Some livers also show mineralization of scattered necrotic hepatocytes. The primary necrotic foci are later infiltrated by histiocytes, lymphocytes and neutrophils, many with marked pyknosis and karyorrhexis. Intracytoplasmic Councilman-like bodies may be present in degenerate hepatocytes or free in sinusoids. Eosinophilic inclusion bodies are often found in the nuclei of cells that are still recognizable as hepatocytes. In older animals, the hepatic necrosis may be less extensive and confined to focal areas of individual lobules, (FAO, 2003).

#### **1.6. Immunity:-**

IgM antibodies first appear three to five days after the onset of RVF clinical signs, at which time viraemia ceases. They persist for one to two months, or even three to four months in some animals. IgG antibodies appear 10-14 days after the onset of infection and persist for at least one to two years or for life. Convalescent immunity after natural infection lasts for a long time. The offspring of immune mothers may have passively acquired maternal immunity for the first three to four months of their lives, (FAO, 2003).



## **1.7. Diagnosis of Rift Valley Fever:-**

### **1.7.1. Field Diagnosis:-**

RVF epizootics should always be strongly suspected when there is a sudden onset of large numbers of abortions in sheep, goats, cattle or camels and deaths in lambs, kids or calves. This is specially the case if there is surface flooding in savannah or semi-arid areas following prolonged rains or in irrigated areas, if the mosquito populations are high; and if there is concurrent illness in human populations, (FAO, 2003). The disease in domestic animals may only be noticed after the illness in people has been identified as RVF. There may also be sporadic cases or small outbreaks in non-epidemic circumstances, which are more difficult to diagnose in the field and may therefore be missed. (FAO, 2003)

### **1.7.2. Histopathology:-**

The finding of characteristic histological lesions with pan-necrosis in the livers of young animals or fetuses is suggestive for RVF.

### **1.7.3. Laboratory Diagnosis:-**

#### **1.7.3.1. Collection and Transport of Samples:-**

Whole blood, liver, lymph nodes and spleen are the tissues of choice for isolation of the virus. Blood samples should be collected from febrile animals into ethylene-diamine-tetra-acetic acid (EDTA) or heparin to which antibiotics have been added, (penicillin 200 units and streptomycin 200 µg/ml, final concentration). Samples of liver and spleen should be collected aseptically both from freshly dead animals at autopsy and from aborted fetuses, if available, and

placed in sterile containers. Duplicate tissue specimens should be collected in neutral buffered formalin for histopathology. Blood samples, about 20 ml each, should be collected from animals in the acute and convalescent phases of the disease, (FAO, 2003).

#### **1.7.3.2. Virus Isolation and Identification:-**

It is recommended that work with this agent should be conducted only in Biosafety Level three facilities, which provide for High Efficient Particulate Air (HEPA) filtration of all exhaust air prior to be discharged from the laboratory, (Sall *et al.*, 1999).

Gear (1988) stated that RVF presents a hazard to all laboratory workers engaged in its study, and appropriate precautions should be taken to prevent infection. These include wearing protective clothing including, at the minimum, goggles, mask, waterproof apron, and gloves. The handling of the virus should be carried out under a hood so designed that the air flows away from the worker to the exhaust, and on the way passes through a battery of ultraviolet lights and a heated chimney. Laminar-flow hoods which achieve the same purpose are standard equipment in most laboratories

It is worth mentioning that conventional virus isolation procedures will remain important for recovery of an infectious virus and for understanding the biology and ecology of the virus and to study the genetic diversity of different strains of RVFV, as re-assortment (exchange of genomes between different strains of RVFV) is likely to occur in nature (Sall *et al.*, 1999).

Various laboratories have different methods for virus isolation (VI); RVF virus can be isolated from whole blood or homogenates

of fresh tissues by intracerebral inoculation of suckling mice or intraperitoneal inoculation of adult mice or hamsters. The identity of the isolated virus is confirmed by polymerase chain reaction (PCR), enzyme linked immunosorbent assay (ELISA), fluorescent antibody staining or virus-serum neutralization tests.

African green monkey kidney (Vero) cells or baby hamster kidney-21 (BHK-21) cells are used for propagation of RVFV. Complete cytopathic effect (CPE) usually occurs in 3-5 days (Eisa *et al.*, 1977). But final result requires 2-4 weeks; the serotype-specific identification of the virus is usually based on serum neutralization test using specific hyper immune serum (Eisa *et al.*, 1977).

#### **1.7.4. Organism Detection Tests:-**

##### **1.7.4. 1. Electron Microscopy and Fluorescent Test:-**

RVF virus attained an extracellular titer of at least 3.6 logs/ml 4 hours post infection in CV-1, Vero and BHK-21 cells. At 22 hours post infection, a peak titer of 7.7 logs/ ml was reached in CV-1 cells, where 50% of the cells showed cytopathic effect. The same degree of cytopathic effect was only observed 45 hour post infection in the other cell lines tested. At 22 hours post infection, RVF viral antigens were detected by indirect fluorescent in the three culture systems; however, the degree of fluorescence and the number of fluorescent cells were much greater in CV-1 than in either Vero or BHK-21 cells. Virus particles were detected by EM, 22 hours post infection in CV-1 cells, but in Vero and BHK-21 cell, the virus could be detected only at 45 hours post infection, (Mekki

and Van Der Groen, 1981). Specific identification of RVF antigen has been achieved in post-mortem human liver by immunodiffusion and in animal tissues by immunofluorescent (Shepherd, 1988).

#### **1.7.4. 2. Animal Inoculation:-**

Morrill *et al*, (1989) stated that Rhesus monkeys inoculated with Rift Valley fever (RVF) virus provide a model in which serial observations of serum viral antigen and antibodies can be made. In 9 non-fatal and 3 fatal infections, either antigen or IgM enzyme-linked immunosorbent assay (ELISA) antibodies were detected in every serum sample during the acute phase. Furthermore, viral nucleic acid could be detected by filter hybridization in most samples taken on days 1 to 3. Circulation of significant quantities of viral RNA provides an additional approach to the diagnosis and study of RVF.

#### **1.7.5. Serological Techniques:-**

Serology may not identify an active infection and cross-reactions at the serogroup level which are likely to occur with other members of the phlebovirus genus. However, it is useful to determine past infection in a seroepidemiological survey, (Aradaib and Abbas, 1985). Several serodiagnostic techniques have been validated for the diagnosis of RVFV. The Agar-gel immunodiffusion test (AGID), Complement fixation test (CFT), Enzyme linked immunosorbent assay (ELISA), hemagglutination (HA) and hemagglutination inhibition (HAI) tests are routinely used (Aradaib and Abbas, 1985). The use of monoclonal antibodies (Mab) in competitive ELISA (cELISA) technique has improved sensitivity and specificity at the serogroup level (Aradaib *et al.*, 1994), this technique

is applicable only to blood (serum) and requires at least 14 days post infection. The ELISA test has now replaced the older haemagglutination inhibition (HI), immunofluorescence assay (IFA) and serum neutralization tests as the test of choice. ELISA systems are available to test for the presence of IgM and IgG, which are extremely valuable in epidemiological investigations. The virus serum neutralization test in microtitre tissue culture systems is still the definitive test system. It is highly specific with little or no cross-neutralization with other phleboviruses. It can be used to detect antibodies in all animal species. However, as it requires the use of live virus, it is not recommended for use outside endemic countries unless a high level of biocontainment is available in laboratories (Sall *et al.*, 1999).

#### **1.7.5.1. Sandwich and Capture ELISA:-**

Paweska *et al.* (2003) reported the development and validation of sandwich and capture ELISAs (both based on inactivated antigen) for detection of IgG and IgM antibody to Rift Valley fever virus in bovine, caprine and ovine sera. Compared to virus neutralisation and haemagglutination-inhibition tests, the IgG sandwich ELISA was more sensitive in detection of the earliest immunological responses to infection or vaccination with Rift Valley fever virus. Its sensitivity and specificity derived from field data sets ranged in different ruminant species from 99.05 to 100% and from 99.1 to 99.9%, respectively, (Paweska *et al.*, 2003). The specificity of IgM-capture ELISA varied between different species from 97.4 to 99.4%; its sensitivity was 100% in sheep tested 5-42

days post-infection. The results in field-collected, experimental and post-vaccination sera demonstrated that these assays might be useful for epidemiological surveillance and control programmes, import/export veterinary certification, early diagnosis of infection, and for monitoring of immune response in vaccinated animals. As highly accurate and safe tests, they have the potential to replace traditional diagnostic methods, which pose biohazard risks limiting their use outside of endemic areas to high containment facilities. Serological techniques mentioned previously, despite their advantage in detecting infected individuals, are complicated by cross-reactions at the level of serogroups and serotypes. To address these problems, molecular diagnostic techniques have been developed.

#### **1.7.5.2. Antigen detection:-**

The RVF antigen may be detected by direct or indirect immunofluorescence tests on impressions smears or cryostat sections of liver, spleen and brain. A rapid diagnosis can sometimes be made by agar gel immunodiffusion (AGID) tests on fresh tissues. Immunocapture-ELISA and histochemical staining of cryostat sections or formalin fixed tissues and PCR are now much more widely used for RVF.

#### **1.7.6. Nucleic Acid Detection Tests:-**

##### **1.7.6. 1. Detection of viral genetic material:-**

A reverse transcriptase PCR test is now available for detection of viral genetic material. Sequencing of the NS (S) protein-coding region of the genome may be used for phylogenetic analysis (genetic fingerprinting) of virus isolates, (Sall *et al.*, 2001).

#### **1.7.6.2. Reverse Transcriptase Polymerase chain Reaction (RT-PCR)**

RT-PCR is important diagnostic tool for rapid detection and differentiation of RVFV infections during endemicity of the disease; it has been successfully applied for detection of RNA viruses by addition of a complementary DNA (cDNA) synthesis step using reverse transcriptase enzyme. (Aradaib *et al.*, 1994).

#### **1.7.6.3. Real Time RT- PCR:-**

Viral hemorrhagic fevers (VHFs) are acute infections agents with high case fatality rates. Important VHF agents are Ebola and Marburg viruses (MBGV/EBOV), Lassa virus (LASV), Crimean-Congo hemorrhagic fever virus (CCHFV), Rift Valley fever virus (RVFV), dengue virus (DENV), and yellow fever virus (YFV). VHFs are clinically difficult to diagnose and to distinguish; a rapid and reliable laboratory diagnosis is required in suspected cases. Six one-step, real-time reverse transcription-PCR assays for these pathogens have been established, (Drosten *et al.*, 2002), based on the Superscript reverse transcriptase-Platinum Taq polymerase enzyme mixture. Novel primers and/or 5'-nuclease detection probes were designed for RVFV, DENV, YFV, and CCHFV using the latest DNA database entries. PCR products were detected in real time on a Light Cycler instrument by using 5'-nuclease technology (RVFV, DENV, and YFV) or SybrGreen dye intercalation (MBGV/EBOV, LASV, and CCHFV). (Drosten *et al.*, 2002). The inhibitory effect of SybrGreen on reverse transcription was overcome by initial immobilization of the dye in the reaction

capillaries. Universal cycling conditions for SybrGreen and 5'-nuclease probe detection were established. Thus, up to three assays could be performed in parallel, facilitating rapid testing for several pathogens. All assays were thoroughly optimized and validated in terms of analytical sensitivity by using in vitro-transcribed RNA. The 95% detection limits as determined by probit regression analysis ranged from 1,545 to 2,835 viral genome equivalents/ml of serum (8.6 to 16 RNA copies per assay). The suitability of the assays was exemplified by detection and quantification of viral RNA in serum samples of VHF patients (Drosten *et al.*, 2002).

**Primers:**

**RVS and RVAs**

**Forward:** RVS AAAGGAACAATGGACTCTGGTCA [349-371]

**Reverse:** RVAs CACTTCT TACTACCATGTCCTCCAAT [443-417]

**Product: Size:** 94 bp

**1.7.6.4. RT- PCR:-**

Sall *et al.*, (2001), recorded that single tube or a nested reverse transcriptase polymerase chain reaction (RT-PCR) method focusing on the NSs coding region of the S segment was developed and used to detect the RVF virus (RVFV) genome, resulting respectively in the synthesis of 810 and 662 bp DNA amplifiers. The assay was specific for RVFV and did not amplify any other phleboviruses known to circulate in sub-Saharan Africa. When serial dilutions of RVFV were artificially mixed with human normal serum, the minimal detection limits were 50 and 0.5 plaque forming



units respectively using the simple and the nested RT-PCR. The RT-PCR method was efficient for the detection of RVFV RNA in the blood from experimentally RVFV-infected mice and lamb and the nested RT PCR was found more sensitive than the virus isolation method. Additionally, this detection method was applied successfully for the diagnosis of human cases during the 1998 Mauritanian outbreak.

**Primers:**

**NSca and NSng**

**Forward:** NSca primer 5'-CCTTAACCTCTAATCAAC-3'

**Reverse:** NSng 5'-TATCATGGATTACTTTCC-3'

**NS3a and NS2g**

**Forward:** NS3a 5'-ATGCTGGGAAGTGATGAGCG-3'

**Reverse:** NS2g 5'-GATTTGCAGAGTGGTCGTC-3'

**Product Size:** 810 bp

**1.7.6.5. RT -PCR used in Mosquito:-**

A reverse transcriptase-polymerase chain reaction (RT-PCR) assay to detect Rift Valley fever (RVF) virus RNA in experimentally infected mosquitoes was developed. The specificity of the assay was evaluated with three other phleboviruses namely; sandfly fever Sicilian (Sabin), sandfly fever Naples (Sabin) and Punta Toro (MSP 3) viruses. The relative sensitivity of the assay, determined by using RVF virus RNA extracted from serial dilutions of virus culture, was approximately 50 plaques forming units. This sensitivity level was 100-fold higher when a nested PCR procedure was used. When the RT-PCR assay was used with coded samples of

intrathoracically-infected and uninfected mosquito, the assay detected the virus in all infected mosquitoes. With this assay, it was possible to detect RVF virus RNA in a single infected mosquito in the background of 10, 25 or 50 uninfected mosquitoes (Ibrahim *et al.*, 1997).

**Primers:**

**RVF1 and RVF2**

**Forward:** RVF1 777/ 5' GAC TAC CAG TCA GCT CAT TAC C 3'/798

**Reverse:** RVF2 1327/5' TG TGA ACA ATA GGC ATT GG 3'/1309

**Product Size:** 551 bp

**RVF3 and RVF4**

**Forward:** RVF3 876/5' CAG ATG ACA GGT GCT AGC 3'/893

**Reverse:** RVF4 1249/5' CT ACC ATG TCC TCC AAT CTT GG 3'/1228

**Product Size:** 374 bp

**1.7.7. Differential Diagnosis:-**

Different members of the arthropod-borne viruses may produce clinical signs and pathological lesions indistinguishable from those induced by RVFV. These viruses include bluetongue virus (BTV) and epizootic hemorrhagic disease Virus (EHDV). BTV and EHDV are closely related members of the orbivirus genus in the family Reoviridae, (Borden *et al.*, 1971). BTV is primarily a disease of sheep and some breeds of deer. Evaluation of a multiplex RT-PCR for simultaneous detection and differentiation of North

American serotypes of BTV and EHDV was developed very recently (Aradaib *et al.*, 2000). Bovine ephemeral fever and Wesselsborne disease may also be confusing during RVFV epizootic (Blood *et al.*, 2000). Malignant ovine theileriosis (*Theileria lestoquidri*) and some bacterial diseases may also confuse diagnosis.

### **1.8. Prevention and Control:-**

The investigation for the focus of the infection is very crucial in the control of the disease. Human and veterinary hospitals and clinical centers offer an excellent opportunity of establishing community relations and furthering social awareness of the impact of communicable diseases that occur in the locality. This information is cheaper than survey. Database should be established in these hospitals and clinics to assist in tracing the environmental source of the emerging viral pathogen or the disease and hence plays an important role in understanding their natural history (Aradaib and Abbas, 1985). In Sudan, RVFV epizootic was first described in 1973 at Kosti, the White Nile Province. Since then, no epizootic has been reported in the Sudan until June of 1976. At that particular point in time, an epizootic of RVFV was reported in Kuku dairy farm, Khartoum North, Sudan. The virus was isolated from a Kenana cow in that farm brought from the same locality where the first RVFV epizootic was reported (Eisa *et al.*, 1980). This information will help in tracing the source of the disease and hence identifying the critical control point.

Importation of animals from endemic areas to RVFV-free areas should be restricted to vaccinated animals to prevent further

dissemination of the virus during RVFV epizootic, especially when it is recognized that vaccination during epizootic is not a valid option. Moreover, an attenuated strain of RVFV is probably circulating in Kuku, Central Sudan.

Prevention and control of RVFV infections are dictated by climatic conditions. Control of the disease by destruction of vector species of the mosquito or the insect is expensive, extremely difficult, and is not a valid option in a vast country, like the Sudan, specially when it is recognized that reintroduction of the vectors via the wind from the surrounding territories is likely to occur. The control measures should be aimed at minimizing contact between susceptible animals and vectors, particularly during RVFV seasons. The use of insect-secured houses and insect repellents for susceptible ruminants should be considered during the flight time of the vector (Aradaib *et al.*, 1999).

#### **1.8.1. Prevention:-**

Pittman *et al.* (1999) stated that, to protect animals and humans from contracting RVF virus, a formalin-killed RVF virus vaccine (P-MKC) from monkey kidney cells infected with the pantropic strain of virus was developed. The prepared vaccine (NDBR-103) was evaluated in mice and hamsters for immune response and efficacy and for its immune response in humans. As applied technologies have been improved, a more modern inactivated product TSI-GSD-200 was developed and after successful preclinical evaluation tested in humans (Pittman *et al.*, 1999). The immunogenicity and safety profiles of TSI-GSD-200 are

excellent. The vaccine protects laboratory workers or others at high risk for RVF disease such as veterinarians in endemic areas. Booster injections can recall immunity lost after the primary series and can elicit antibodies in those with a sub-optimal response to the initial immunizations, Pittman et al., (1999).concluded, the use of TSI-GSD-200 is safe and provides good long-term immunity in humans when the primary series and one boost are administered (Pittman et al., 1999). RVF vaccination is not recommended for the average traveler, but although it is not generally available, it may be indicated for those who are traveling to participate in international RVF-outbreak investigations or are otherwise at high risk of exposure (Isaacson, 2001).

#### **1.8.2. Control of RVF in Vertebrate Hosts:-**

Immunization of susceptible animals is the most effective means for control of RVF (Meegan and Bailey, 1989). Two types of vaccines are currently used to immunize sheep and cattle in Africa. The attenuated live virus vaccine (Smithburn strain) is highly effective, but causes a small number of sheep to abort after immunization. It is relatively inexpensive and has been extensively used in endemic areas. Since there is a theoretical potential for reversion of the vaccine to virulence, it is not recommended for non-endemic areas or for animals being exported from endemic areas (Meegan and Bailey, 1989). Formalin-inactivated vaccines have been used for many years in southern Africa, and recently in Egypt and Israel. These are safe and effective, but require multiple inoculations. This type of vaccine is recommended for non-endemic

areas or for animals being exported from endemic areas. Attenuated vaccine strains with several attenuation markers present in each genome segment should be considered as a safety measure for control of RVFV and to minimize the possibility of reversion to virulence (Sall *et al.*, 1999).

An innovative subunit vaccine expressing the nucleocapsid protein, which is coded for by the small genome (S) genome, was recently evaluated in mosquito cells. The expressed recombinant antigen vaccine induced pathogen-specific resistance to RVFV in mosquito cells (Billecocoq *et al.*, 2000). In general, the subunit vaccine requires boosting and adjuvant to provide long lasting protective immunization (Aradaib *et al.*, 1995). The research on development of this subunit vaccine is in progress but the vaccine is not yet available for animal or human use. A broad-spectrum vaccine, which can protect against all RVFV strains, is yet to be developed.

#### **1.8.2.2. Quarantine Measure:-**

Since enforcement of quarantine of animals is difficult in Africa, it is not generally an effective control measure. However, to whatever extent possible, movement of animals from epizootic situations should be restricted to prevent the further spread of RVF (Meegan and Bailey, 1989).

#### **1.8.3. Mosquitoes avoidance:-**

A person's chances of becoming infected can be reduced by taking measures to decrease contact with mosquitoes and other

bloodsucking insects through the use of mosquito repellents and bednets (website 4)

#### **1.8.4. Avoidance of exposure to blood or tissues of animals:-**

Avoiding exposure to blood or tissues of animals that may potentially be infected is an important protective measure for persons working with animals in RVF-endemic areas (website 4)

#### **1.8.5. Treatment:-**

Treatment is supportive and may require intensive care. Early diagnosis and supportive care can be lifesaving for most patients with VHF.(Anon.1988), The cornerstone of therapy for all these infections is judicious fluid and electrolyte management (website 3 ). Patients with RVF should be nursed in mosquito-protected premises (Isaacson, 2001). There is no established course of treatment for patients infected with RVF virus. However, studies in monkeys and other animals have shown promise for Ribavirin, an antiviral drug, for future use in humans. Additional studies suggest that interferon, immune modulators, and convalescent-phase plasma may also help in the treatment of patients with RVF (Website 2).

#### **1.9. Records of Epizootic or Infected Individuals:-**

The attending clinician should report in a case summary form the information about the clinical signs of the disease, tentative diagnosis, prescribed medicine, and date of discharge as routinely done in humans and veterinary hospitals or clinics. If the patient died, postmortem findings and pathological lesion should be recorded, as they might be of significance in tentative diagnosis of RVFV infection. A similar procedure should be followed, if the

disease occurs in an epizootic form in animals and human populations. The place where the disease appears should be recorded. If an epizootic of hemorrhagic disease is suspected, notification of the authority would be the responsibility of the medical or veterinary officers in charge.

A collaborative scientific team should immediately conduct research investigation to implement hazard analysis and critical control point (HACCP) system to prevent further spread of the disease. RT-PCR would be advantageous for detection of an active RVFV infection within the same working day. Morbidity, mortality and case fatality rates are useful to estimate the economic significance of the epizootic. The season of the year is important for understanding the mode of transmission, where the vector density is usually very high in the rainy seasons. It should be considered that, overwintering transmission is common despite the fact that the activity of the invertebrate vectors is likely to be suppressed. This might help in identifying other reservoir hosts of the RVFV if the epizootic occurs in the winter. This information should be recorded and kept for future purposes.



## **CHAPTER TWO**

### **2-MATERIALS AND METHODS**

#### **2.1. Source of samples suspected RVF:**

During September, 2002 an unusual heavy rainfall hit most regions of Sudan. This led to an increase in mosquitoes vectors. A febrile illness spread among cattle in Khartoum State; East Nile Province, in Almaigoma, Hillat Kuku, Abu Defiaea, Eid Babiker Babiker, Mahalab 1 and 2, Alhaj Yousif (Fig.1) almost every locality in the Province was affected. Calves were severely affected with approximately 20-50% morbidity and in some herds, mortality reached almost 70%, Sheep exhibited less severe clinical signs. The disease incubation period was 3-7 days. Most of the pregnant animals aborted, and there was a sharp decrease in milk yield. In October the same year the disease extended to Khartoum South in Alshajara, military farm TaybaAlhassanab, Butry and Traiatalbija, with the same nature of high abortion rates and off-spring mortality. No mortality was recorded in mature animals. Clinical signs observed in diseased animals were salivation, nasal discharge, lacrimation, abortion, retained placenta, drop in milk production. In aborted feti there was enlargement of the head and abdomen. (Table .1). During this period, the same picture of the disease was reported in the River Nile State in Shendi, Alsayal, Aljiware and some other villages. In some farms there was malaria-like disease observed in humans who were in close contact with animals. By mid November the disease almost died out.

## **2.2. Study Design:-**

Serosurveillance for RVFV antibodies was made during the year 2001, investigation of the suspected RVF outbreak in Khartoum and River Nile States was studied in the animals affected during 2002.

## **2.3. Animal Sera:-**

Sera were collected from cattle, goats and sheep from different geographic localities in the Sudan during 2001. They were obtained from the Department of Epizootics Control, Federal Ministry of Animal Resources and Fisheries. Other sera and diagnostic specimens (liver, spleen and lymph node from aborted fetuses) were collected from Khartoum, River Nile and White Nile states during 2002 and 2005 were also used in the study, sera were kept at -20 till used.

## **2.4. RVFV Vaccine Strain (Smithburn):-**

Smithburn live attenuated vaccine strain was imported from South Africa kindly donated to us by the Viral Vaccines Department, at the Central Veterinary Research Lab. (CVRL-Soba).

## **2.5. Hemorrhagic RNA Viruses:-**

Bluetongue virus (BTV), epizootic hemorrhagic disease virus (EHDV) and Crimean Congo hemorrhagic fever virus (CCHEV) RNAs were kindly provided by Prof Imad Aradaib, Department of Medicine Pharmacology and Toxicology, Faculty of Veterinary Medicine, University of Khartoum.

## **2.6. African Green Monkey Kidney Cell (Vero cell):-**

Vero cells were kindly obtained from the viral vaccines Production Section (CVRLC), and used for the propagation of the vaccine strain.

**2.7. Detection of RVFV Antibodies and Antigen using different Techniques includes:-**

- 2.7. 1. Indirect Sandwich ELISA: used for anti RVFV IgG detection
- 2.7.2. Immunocapture ELISA: used for anti RVFV IgM detection
- 2.7.3 Indirect ELISA: used for RVFV antigen detection.
- 2.7.4. Indirect fluorescent antibody test (IFAT)
- 2.7.5. Reverse transcriptase polymerase chain reaction (RT-PCR)

### **2.7.1. Indirect Sandwich ELISA for the Detection of anti-RVFPV IgG Antibody:-**

Commercial ELISA kits for determination of RVFPV IgG antibodies were purchased from the special pathogen unit, National institute for Virology, Johannesburg, South Africa .The ELISA is based on a sandwich format in which the plate was coated with mouse anti-RVF serum and then reacted with antigen. Test sera were applied and specific anti-RVFPV IgG antibody was detected with an anti-species IgG HRPO conjugate and ABTS substrate.

#### **2.7.1.1 Chemicals used for Indirect Sandwich ELISA:-**

Deionized distilled water(DDW), Phosphate buffered saline (PBS 0.01M PH 7.4), skimmed milk, Tween 20 (T20), washing buffer(PBS, 0.1% T20), blocking buffer( 10% skimmed milk in PBS) diluent buffer (2% skimmed milk in PBS). 2, 2-Azinodichthyl-benzothiazoline sulfonic acid (ABTS) substrate, and Sodium dodecylsulphate (SDS) stop solutions.

#### **2.7.1.2. Biological reagents for Indirect Sandwich ELISA:-**

Mouse anti-RVFPV serum (coating antibody), RVFPV antigen, control antigen, rabbit anti-sheep horseradish peroxidase (HRPO) conjugate, control sera: strong positive (C++), weak positive (C+) and negative control serum (C-).

#### **2.7.1.3. Test Procedure:-**

The test was conducted as described by the manufacturers; all the solutions were prepared immediately before use. As shown in figure (2) the microtitre plate (Nunc maxisorp) was coated with 100 µl /well anti- RVFPV serum diluted 1:5000 in PBS (0.01M, Ph 7.4)

.The plate was covered with lid ,incubated at 4°C overnight, and then emptied, washed three times with washing buffer, then blocked with 200 µl/well skimmed blocking buffer, while being incubated in moist chamber at 37°C for 1hour. The plate was then emptied and again washed before 100 µl/well of RVFV antigen and control antigen diluted 1:400 in diluent buffer were added from A to D, 1-12 rows and from E to H, 1-12 rows respectively .The plate was incubated then washed as described before 100 µl volumes of the control and tested sera freshly diluted 1:400 diluent buffer were then delivered into duplicate wells of the plate, Column 1 and 2 were used as control columns, while the rest of the plate for the tested sera .The plate was again incubated and washed. 100 µl/well Anti-sheep IgG HRPO conjugate diluted 1:5000 in diluent buffer was added to all wells of the plate. Then the plate was incubated as described before, then washed 3 times.100 µl of ABTS substrate was then added to all wells and the plate was kept for 30 minutes in a dark place at room temperature. The reaction was then stopped by adding 100 µl/well of 0.1% Sodium dodecyl sulphate (SDS)) stop solutions. The plate was read in Immunoskan (BDSL,United Kingdom) ELISA plate reader using 405 nm filter.

### **2.7.2. Immunocapture ELISA for the detection of anti-RVFV IgM**

#### **Antibody (BDSL-UK):-**

Commercial ELISA kits for determination of RVFV IgM antibody were purchased from Biological Diagnostic Supplies (BDSL; United Kingdom). The ELISA is based on a capture format

in which the plates were coated with rabbit anti-sheep IgM capture antibody and then reacted with test sera. Anti sheep capture antibody can be used for detection of IgM in sheep, goats and cattle. The captured IgM antibody was reacted with RVF antigen, and the bounded antigen was then detected with mouse anti-RVF antibody anti-mouse horseradish peroxidase (HRPO) conjugate plus ABTS substrate.

#### **2.7.2.1. Chemicals for Immunocapture (ELISA):-**

Deionized distilled water(DDW)Phosphate buffered saline (PBS 0.01M PH 7.4), skimmed milk, Tween 20 (T20), washing buffer(PBS, 0.1% T20), blocking buffer( 10% skimmed milk in PBS) diluent buffer (2% skimmed milk in PBS) 2, 2-Azinodichthyl-benzothiazoline sulfonic acid (ABTS) substrate, and Sodium dodecylsulphate (SDS) stop solutions.

#### **2.7.2.2. Biological reagents for Immunocapture ELISA:-**

Rabbit anti sheep IgM, RVFV antigen, control antigen mouse anti-RVFV serum, Goat anti-mouse IgG horse-radish peroxidase (HRPO) conjugate, control sera: strong positive (C++), weak positive (C+) and negative control (C-)

#### **2.7.2.3. Test procedure:-**

The test was performed according to the manufacturers protocol, all the solutions were prepared immediately before use as shown in figure.3.the microtitre plate (Nunc Maxisorp) was coated with 100 µl /well RVFV rabbit anti-sheep IgM diluted 1:2000 in PBS .The plate was incubated at 4°C overnight, emptied and washed three times with washing buffer, then blocked with 200 µl /well

blocking buffer and incubated in moist chamber at 37°C for hour. The plate was then emptied and again washed as before. 100 µl volumes of the control and tested sera freshly diluted 1:400 in diluent buffer, were delivered into duplicate wells of the plate, Columns 1 and 2 were used as control columns, while the rest of the plate was used for the test sera. The plate was again incubated and washed as previously described. 100 µl/well of RVFV antigen and control antigen freshly diluted 1:400 in diluent buffer were added from A to D, 1-12 rows and from E to H, 1-12 rows respectively. The plate was incubated again, then washed as above, 100 µl of Mouse anti-RVFV serum diluted 1:500 in diluent buffer was added to each well, The plate was incubated for 1 hour and then washed, 100µl/well anti-mouse IgG horseradish peroxidase (HRPO) conjugate freshly diluted 1:4000 in diluent buffer was added to all the plate, another incubation was done as described before, then emptied and washed again, 100 µl of ABTS substrate were added to all wells and the plate left for 30 minutes in a dark place at room temperature. The reaction was then stopped by adding 100 µl/well of 0.1% Sodium dodecyl sulphate (SDS)) stop solutions. The plate was read in Immunoskan (BDSL, United Kingdom) ELISA plate reader using 405 nm filter.

### **2.7.3. Indirect ELISA for detection of RVFV antigen:-**

This ELISA was formulated and developed at CVRL to detect RVF antigen from suspected tissue samples and sera collected during the period of 2002. The ELISA is based on indirect way in

which the plate was coated with RVF Ag which was then reacted with mouse anti- RVFV antibody and anti-species IgG HRPO conjugated and ABTS.

#### **2.7.3.1. Chemicals used for Indirect ELISA:-**

Deionized distilled water(DDW) Phosphate buffered saline (PBS 0.01M PH 7.4), skimmed milk, Tween 20 (T20), washing buffer(PBS, 0.1% T20), blocking buffer( 10% skimmed milk in PBS) diluent buffer (2% skimmed milk in PBS) 2, 2-Azinodichthyl-benzothiazoline sulfonic acid (ABTS) substrate, and Sodium dodecylsulphate (SDS) stop solutions.

#### **2.7.3.2. Biological reagents used for Indirect ELISA:-**

RVFV antigen, control antigen, Mouse anti-RVFV serum, Anti-mouse IgG horseradish peroxidase (HRPO) conjugate, all of these reagents were obtained from the Biological Diagnostic Supplies Ltd. (BDSL) United Kingdom (UK)

#### **2.7.3.3. Test procedure:-**

All the solutions were prepared immediately before use as shown in figure .4. The microtitre plate (Nunc Maxisorp) was coated with RVFV Ag diluted 1:400 in diluent buffer, columns 1 and 2 were used as control Ag from A to B for the known +ve Ag and from C to D for the known -ve Ag. The rest of the plate was used for the samples, (from 1-8 serum samples, 9-12 tissue samples, liver and spleen from the oozing fluids), the plate was covered with lid, incubated for 1 hour in moist chamber then washed as described above for both ELISA. 100µl/well Mouse anti RVFV serum diluted 1:500 in diluent buffer were added. The plate



was incubated then washed. Anti-mouse IgG HRPO conjugate diluted 1:4000 in diluent buffer were added to all the plate at the rate of 100µl/well. The plate was incubated in moist chamber at 37°C for 1 hour, and then washed as described before. 100µl of ABTS substrate were added to all wells then the plate was left for 30 minutes in a dark place at room temperature. The reaction was then stopped by adding 100 µl/well of 0.1% Sodium dodecyl sulphate (SDS)) stop solutions. The plate was read in Immunoskan (BDSL) ELISA plate reader using 405nm filter.

**Figure.2. Indirect Sandwich ELISA for the Detection of Anti-RVSV IgG antibody. Plate layout**

	1	2	3	4	5	6	7	8	9	10	11	12
A	C++	C++	1	5	9	13	17	21	25	29	33	37
B	C++	C++	2	6	10	14	18	22	26	30	34	38
C	C+	C+	3	7	11	15	19	23	27	31	35	39
D	C-	C-	4	8	12	16	20	24	28	32	36	40
E	C++	C++	1	5	9	13	17	21	25	29	33	37
F	C++	C++	2	6	10	14	18	22	26	30	34	38
G	C+	C+	3	7	11	15	19	23	27	31	35	39
H	C-	C-	4	8	12	16	20	24	28	32	36	40

C++: Strong control positive serum

C+ : Weak control positive serum

C- : Negative control serum

1-40 test sera

Rows A-D 1-12 RVSV Ag

Rows E-H 1-12 Control Ag

**Figure .3.Immunocapture ELISA for the detection of  
Anti-RVSV IgM antibody Plate layout**

	1	2	3	4	5	6	7	8	9	10	11	12
A	C++	C++	1	5	9	13	17	21	25	29	33	37
B	C++	C++	2	6	10	14	18	22	26	30	34	38
C	C+	C+	3	7	11	15	19	23	27	31	35	39
D	C-	C-	4	8	12	16	20	24	28	32	36	40
E	C++	C++	1	5	9	13	17	21	25	29	33	37
F	C++	C++	2	6	10	14	18	22	26	30	34	38
G	C+	C+	3	7	11	15	19	23	27	31	35	39
H	C-	C-	4	8	12	16	20	24	28	32	36	40

C++: Strong control positive serum

C+ : Weak control positive serum

C- : Negative control serum

1-40 test sera

Rows A-D 1-12 RVSV Ag

Rows E-H 1-12 Control Ag

**Figure.4. Indirect ELISA for detection of RVFV antigen**

**Plate layout**

	1	2	3	4	5	6	7	8	9	10	11	12
A	Ag++	Ag++	5	5								
B	Ag++	Ag++	6	6								
C	Ag-	Ag-	7	7								
D	Ag-	Ag-	8	8								
E	1	1	9	9								
F	2	2	10	10								
G	3	3	11	11								
H	4	4	12	12								

++Ag: Positive control antigen

-Ag : Negative control antigen

1-12 : Test samples

#### *2.7.4. Indirect fluorescent Antibody (IFA) test:-*

##### **2.7.4.1. Chemicals used for (IFA) test:-**

Deionized distilled water (DDW), Phosphate buffered saline (PBS 0.01M PH 7.2), Tris buffer, pH 7.4 acetone (Analar), glycerol mounting media and fluorescein-isothiocyanate (FITC).

##### **2.7.4.2. Biological reagents used for (IFA) test:-**

RVFV vaccine strain(Smithburn), fetal calf serum (FCS), (Sigma USA),rabbit anti-bovine IgG antibody conjugated to FITC, positive and negative RVFV control sera and test sera.

##### **2.7.4.3. Antigen slides preparation:-**

In brief flask cultures of Vero cells were infected with RVF vaccine strain (Smithburn) and the cells were harvested by trypsinization 60 hours post infection at an early stage of cytopathic effect(CPE). The cells were centrifuged at 800 g for 5 minutes and resuspended at  $10^6$ /ml in tris-buffered saline, pH 7.4, containing 5% fetal calf serum (FCS). The cells were held at 4°C overnight, washed and resuspended at  $10^6$ /ml in isotonic saline with 5% FCS. Spots were prepared from 0.01ml volumes of cell suspension on 8-well Teflon-template microscope slides (Highveld Biological (PTY).LTD, Lyndhurst, RSA). This was done by pipetting 0.01ml of the suspension into a well and immediately aspirating the excess and transferring it to the next well using single channel micropipette. The slides were allowed to air-dry, fixed in ice cold acetone for 10 minutes and again air-dried, and then they were individually wrapped in tissue paper and packed in aluminum foil with 5 slides in each packet. These packets were labeled indicating

numbers and date of the preparation of the slides, and then stored at 80°C until used.

#### **2.7.4.4. Conjugate:-**

Rabbit anti-bovine IgG conjugated to FITC was kindly obtained from the department of ticks and tick borne disease in 0.1 ml a aliquot.

#### **2.7.4.5. Dilution of the Conjugate:-**

An aliquot of (0.1 ml) of the conjugate was thawed at room temperature and diluted 1:20 in PBS pH 7.2. Evans blue (50 µl) at a concentration of 0.1% was added to the conjugate as a counterstain in order to improve the definition of specific fluorescence.

#### **2.7.4.6. Control sera:-**

The positive and negative control sera were provided in the ELISA kit as mentioned before.

#### **2.7.4.7. Dilution of the tested sera:-**

Bovine sera collected from the field were allowed to thaw at room temperature, and then arranged in numerical order in a rack; sera were diluted 1:10 dilution in PBS, pH 7.2.

#### **2.7.4.8. Thawing of the antigen slides:-**

Antigen slides were allowed to thaw at 4 °C for 30 minutes then placed at room temperature for another 30 minutes before they were unwrapped and labeled with location and tested sera numbers.

#### **2.7.4.9. Test procedure:-**

The test was conducted as described by (Swanepoel *et al.*, (1986) with slight modification, the antigen slides once thawed and labeled were placed in Petri dishes on a moist filter paper. To each

slide 20µl of C +ve and C -ve sera were added to the well 6 and well 12 respectively, the tested sera were added to the rest of the wells by adding 20µl diluted sera using a single micropipette (Fig.5.). A clean tip was used for each tested serum sample. The slides were incubated in a moist chamber at 37 °C for 30 minutes to allow antigen/antibody reaction, the slides were washed three times, 10 minutes each in stirred PBS, pH 7.2. The buffer being renewed after each 10 minutes, and then the slides were held inverted onto a filter paper for excess PBS to drain. The slides were replaced in a Petri-dish and 10µl of the diluted conjugate was applied to each well and allowed to react for a further 30 minutes at 37°C in moist chamber, again the slides were washed as described above, dried and prepared with glycerol mounting medium and cover-slip for examination on an indirect-light fluorescent microscope in a dark room.

Label	1	2	3	4	5	6 C+ve
A1	7	8	9	10	11	12 C-ve

**Figure.5. Diagrammatic representation of multi-spot slide showing the positions of the controls and tested sera**

### **2.7.5. Reverse transcriptase polymerase chain reaction (RT-PCR):**

Single tube reverse transcriptase polymerase chain reaction (RT-PCR) method focusing on the NSs coding region of the S segment was developed and used to detect the RVF virus (RVFV) genome.

#### **2.7.5.1 Chemicals and Reagents for RT-PCR:-**

QIA amp viral RNA extraction kit contains the following: lyophilized carrier RNA, AVL buffer, pure ethanol, AW<sub>1</sub>, AW<sub>2</sub> washing buffers, and AVE lyses buffer. Single tube RT-PCR kit contains: 5x reaction buffer, 5Mm dNTP mix, pH 7.5 with dATP, dCTP, dGTP, dTTP. Enzyme mix (Taq-DNA-Polymerase+Rnase Inhibitor), RVFV Primers and ddH<sub>2</sub>O.

#### **2.7.5.2. Extraction of Viral RNA:-**

Extraction of viral nucleic acid was carried out by the QIA amp RNA extraction kit (QIA GEN GmbH, Hilden, Germany). As described by the manufacturers. RNAs were extracted from supernatant of Vero cell culture infected with RVFV vaccine strain (Smithburn) and homogenate from aborted feti tissue samples as follows;

a) The lyophilized carrier RNA was dissolved in 1ml AVL buffer and transferred to the AVL bottle and mixed thoroughly. Five hundred and sixty (560) µl from this preparation were dispensed in each 1.5ml micro centrifuge tube and kept at 4°C, till used.



- b) All samples and reagents were equilibrated to room temperature before use.
- (c) 140 µl medium from Vero cell culture infected by RVFV vaccine strain (Smithburn) and homogenate from aborted fetal tissue samples were added to the buffer AVL containing carrier RNA and mixed by pulse vortexing for 15 seconds.
- (d) The mixture was left for 10 minutes at room temperature.
- (e) The tubes were then spun briefly to clean the lid.
- (f) 560 µl of pure ethanol was added and mixed by pulse vortexing for 15 seconds and spinning briefly.
- (g) 630µl from the mixture were applied in the QIA amp spin column and assembled in a 2ml collection tube then centrifuged at 8000 rpm for 1 minute.
- (h) The columns were transferred to another collection tubes; the remaining 630µl of the mixture was applied and centrifuged at 8000 rpm for 1 minute again.
- (i) 500µl of buffer AW<sub>1</sub> was added to the columns and centrifuged at 8000 rpm for 1 minute. Another 500µl of wash buffer AW<sub>2</sub> was added and centrifuged at 14000 rpm for 3 minutes.
- (j) The spin column were transferred to 1.5 ml micro centrifuge tubes and loaded with 60µl of buffer AVE to elute the viral RNA. It was centrifuged at 8000 rpm for 1 minute. The tubes containing RNAs were labeled and kept at -20°C till used.

#### **2.7.5.3. Primers:-**

The primers were kindly donated by Prof. Gerrit J. Viljoen, S. Africa

These primers were flanking 363bp regions in the S segment of the RVFV genome. **Forward:**5'-ATGCTGGGAAGTGATGAGAGCG-3'

**Reverse:** 5'-GATTTGCAGAGTGGTCGTC-3'

#### **2.7.5.4. Reverse transcriptase polymerase chain reaction (RT-PCR)**

The amplification was performed using peqlab single tube RT-PCR kit (GmbH, Germany). The following components were mixed in a PCR reaction tube: 5 µl total RNA, 5µl 5x reaction buffers, 2.5µl 5mMdNTP mix, 2µl primers and 1µl enzyme mix. The final volume was then completed with ddH<sub>2</sub>O to 25µl. The components were mixed thoroughly by vortexing and centrifuged at 8000 rpm for 1 minute. The RT-PCR was performed in Techne PHC-2 thermal cycler (Techne, Princeton, NJ, USA). Initial transcription step was done at 42°C for 30 minutes followed by 35 cycles of amplification. Each cycle include denaturation at 95°C for 10 minutes, annealing at 65°C for 5 minutes and extension at 72°C at 5 minutes.

#### **2.7.5.5. Agarose gel electrophoresis:-**

Following RT-PCR, 15µl of the total reaction volume were added to 5µl of Bromophenol blue dye, loaded to 1% agarose gel and then submerged in Tris Borate EDTA (1X TBE) running buffer containing 0.001% Ethidium bromide(50mg/ml). 2 µl DNA molecular weight marker 1kb ladder was run in a parallel well. Constant electric current of 80 MV for about 45 minutes was applied.

#### **2.7.5.6. Sensitivity of the RT-PCR:-**

To determine the minimal amount of the RVFV vaccine strain that can be detected using simple RT-PCR, the extracted RNA was serially diluted in ddH<sub>2</sub>O ranging from 10<sup>-1</sup> to 10<sup>-6</sup> and one tube was kept with master mix alone i.e. without RNA as negative control. All the tubes were amplified except the control one, as mentioned before and applied on agarose gel.

#### **2.7.5.7. Specificity of the RT-PCR:-**

The specificity of the (RVF) virus primers was tested with other hemorrhagic viruses like, bluetongue virus (BTV), epizootic hemorrhagic disease virus (EHDV) and Crimean Congo hemorrhagic fever virus (CCHFV). Viral RNAs were amplified by single tube RT-PCR and analyzed in agarose gels.

#### **2.8. Entomological Study:-**

Part of this work was conducted at the general Administration of Malaria Control, Ministry of Health, Department of Medical Entomology, National Health laboratory and the CVRL.

Two types of collection were carried out:

1. Larval collection method or dipping collection method for aquatic stages of mosquitoes. Twenty dips were taken at regular intervals from fixed productive sites of mosquitoes from March till November 2002 in Khartoum State. Collected larvae were transferred into plastic containers and protected to minimize shaking.
2. Adults collection: two methods were used for collecting adults mosquitoes indoor spray collection and aspiration collection

methods. In the laboratory, the fourth instar larvae were separated from other stages counted and prepare for identification. For preparation of larval mounts, the larvae were killed in warm water then dehydrated in 70% ethyl alcohol followed by 5 minutes in each 80%, 90% and absolute alcohol, Abugroon *et al.* (1988).

## **CHAPTER THREE**

### **3. RESULTS**

#### **3.1. Detection of RVF antibodies:-**

##### **3.1.1. IgG ELISA:-**

A total of 1351 sera (491 bovine ,423,ovine and 437 caprine) from different regions were screened for RVF IgG antibodies at 1/100 dilutions .Out of these ,15 (3.55%) ovine sera ,and 1 (0.25%) caprine serum were positive while none of the bovine samples tested positive (Fig.7.). The overall prevalence rate was 1.2%. The prevalence rate of suspected RVF IgG antibodies in sheep ranged from 10% in Shendi (River Nile State) to 3.4% in Buram (Southern Darfur State). The one goat serum found to be positive was from Gedarif State.

##### **3.1.2. IgM ELISA:-**

A total of 1543 serum samples collected from three different animal species ( 618 from bovine ,612 from ovine and 313 from caprine ) from different regions(Northern, eastern, western and central Sudan ), were screened for RVF IgM antibodies at 1/100 dilutions, no positive results were recorded as shown in Table .2.and fig.8. During 2002, 107 bovine serum samples were tested for RVF IgM antibodies, 31(28.97%) out of these were found positive, (Fig.6.) Compared to 9(2.7%) serum samples from 410 serum samples from the same species collected during 2005, (Fig.11).

The prevalence rate was found to be 21.1% and 70.5% among bovine samples collected from Khartoum and Shandi respectively during 2002. As shown in (Fig.10.), RVF IgM antibodies were not detected among serum samples collected from both ovine and caprine species. No clinical cases were reported during 2003. Moreover all the positive reactors during 2002 were culled or removed from the herds.

### **3.1.3. Antigen Detection ELISA:-**

Four tissue samples and eight sera were screened for RVF antigen using Indirect ELISA. Two tissues liver and spleen were positive, showing intense color development while the three serum samples were faint.

### **3.2. Results of IFA test:-**

100 bovine serum samples collected from different locations in Khartoum were tested for RVF antibodies using IFA test, of which 31 samples were positive for IgM ELISA. Twenty three samples were positive with IFA test; in comparison with ELISA this test was slightly less sensitive and less specific in detecting RVF antibodies.

### **3.3. Development of the detection methods:-**

Two steps for RT-PCR were tested initial transcription step followed by 35 cycles of amplification. Analysis of the RT-PCR products in agarose gel and staining with promphenol blue revealed the presence of the expected bands of the 363 bp obtained from RVFV vaccine strain and two homogenate of spleen and liver samples of aborted feti RNAs extracts (Fig.12.).

### **3.4. Sensitivity of the RT-PCR:-**

The RT-PCR method is a sensitive test for detection of RVF virus even if a minimal amount of viral RNA is present (Fig.13.).

### **3.5. Specificity of the RT-PCR:-**

RT-PCR product was not detected when RNA samples from BTV, EHDV and CCHF were applied; this indicate that the primers did not amplify any region in the genome of these viruses and this show the specificity of this system for RVFV(Fig.14.).

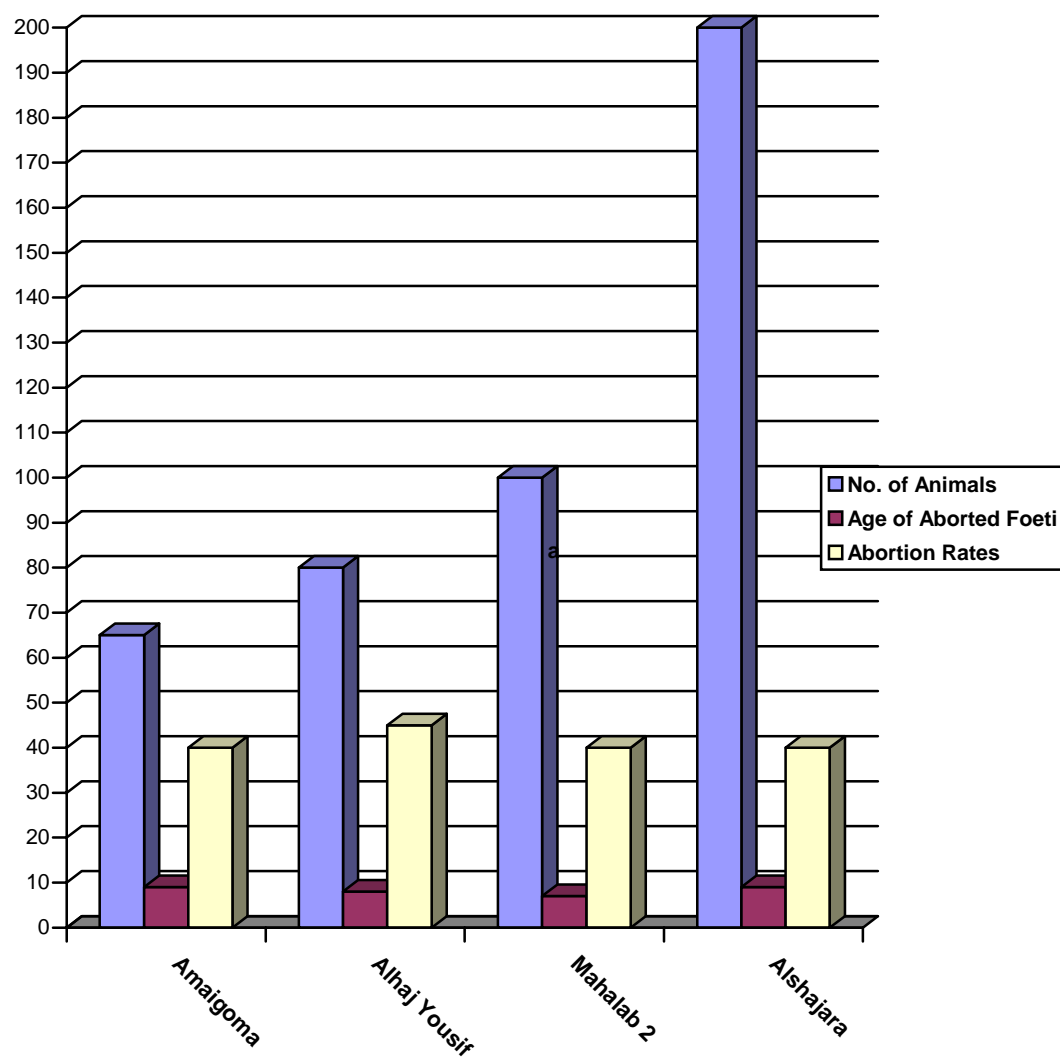
### **3.6. Species Composition:-**

Culicine mosquitoes accounted for 78.4% and *anopheline* for 21.6%. *Culex quinquefasciatus* alone accounted for 81.9% of the mosquitoes of the genus in the capital towns and suburb while *Anopheles arabiensis*, the main malaria vector in the area contributed about 97.2% of the genus *Anopheles*. Among the three cities of the capital Khartoum sustained more species than Khartoum North and Omdurman. In the year 2001 a total of 4353 larvae were collected by dipping method and a total of 725 adults mosquitoes were identified. In the year 2005, a total of 3372 *Anopheles* and 853 *Culex* larvae were collected, all these species of mosquitoes transmit RVFV even *anophelines* the vector of malaria parasite *plasmodium* it causes rupture of the salivary glands of the mosquitoes and then facilitate transmission of the RVF virus.

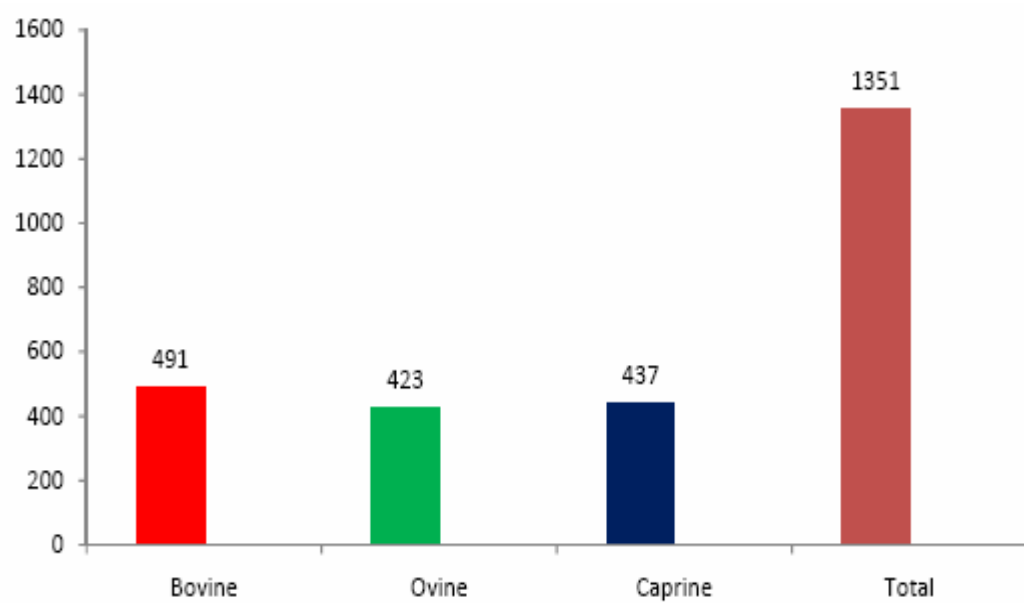
**Table (1) Shows the locality and abortion rates in some  
examined cattle herds in Khartoum State during the year 2002**

Locality	No of animals examined	Age of aborted feti/months	Abortion rates	Type of samples collected
Almaigom a	<b>65</b>	<b>5-9</b>	<b>40%</b>	Whole blood, liver ,vaginal Swap and spleen
AlhajYosif	<b>80</b>	<b>5-8</b>	<b>20-45%</b>	Whole blood, vaginal swap and liver
Mahalab 2	<b>100</b>	<b>5-7</b>	<b>40%</b>	Whole blood, vaginal swap ,liver and spleen
Alshajara	200	<b>5-9</b>	<b>40%</b>	Whole blood ,liver and spleen





**Figure.6. Showed the locality and abortion rates in some examined cattle herds in Khartoum State during the year 2002**



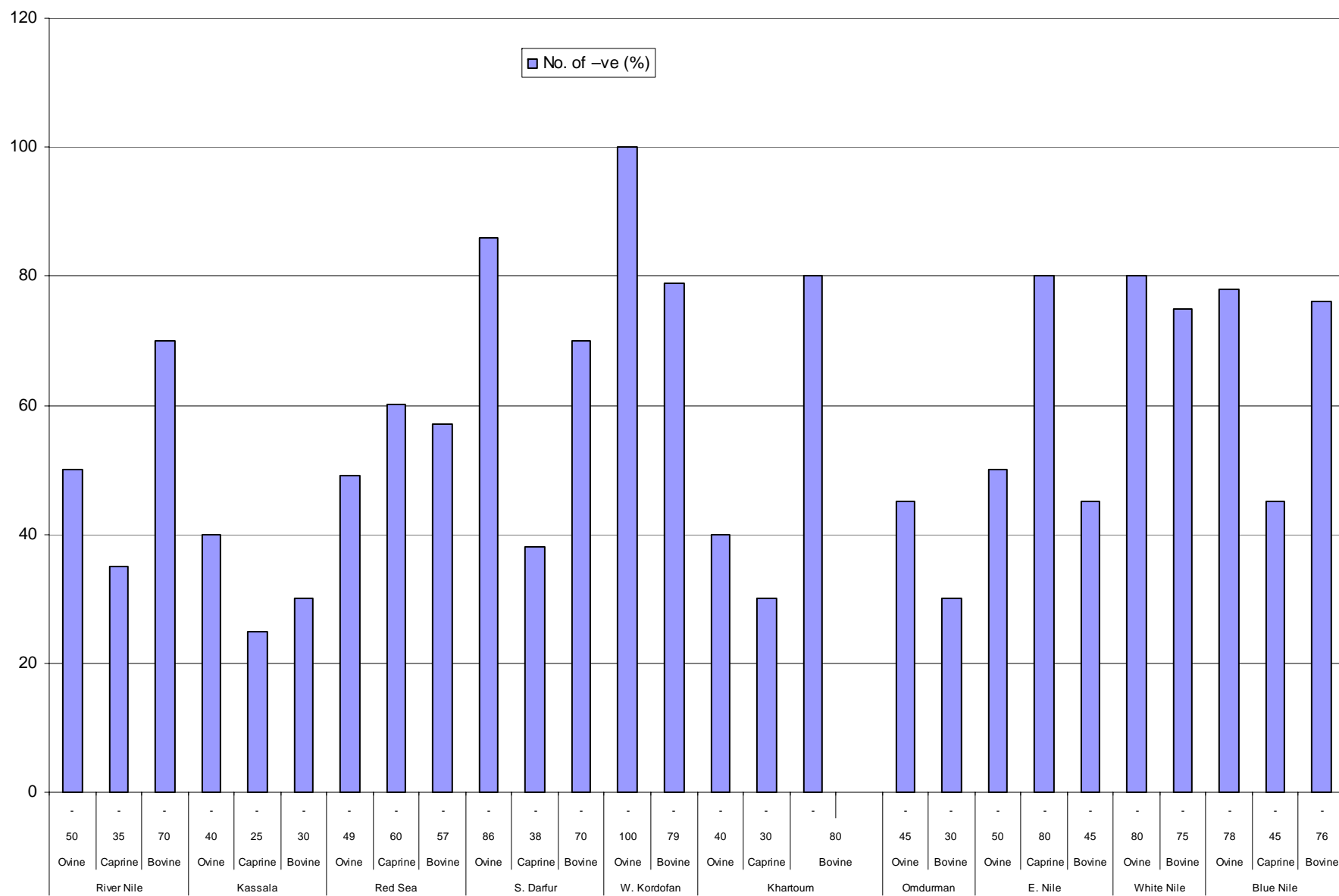
**Figure 1. Result of IgG ELISA (2001)**

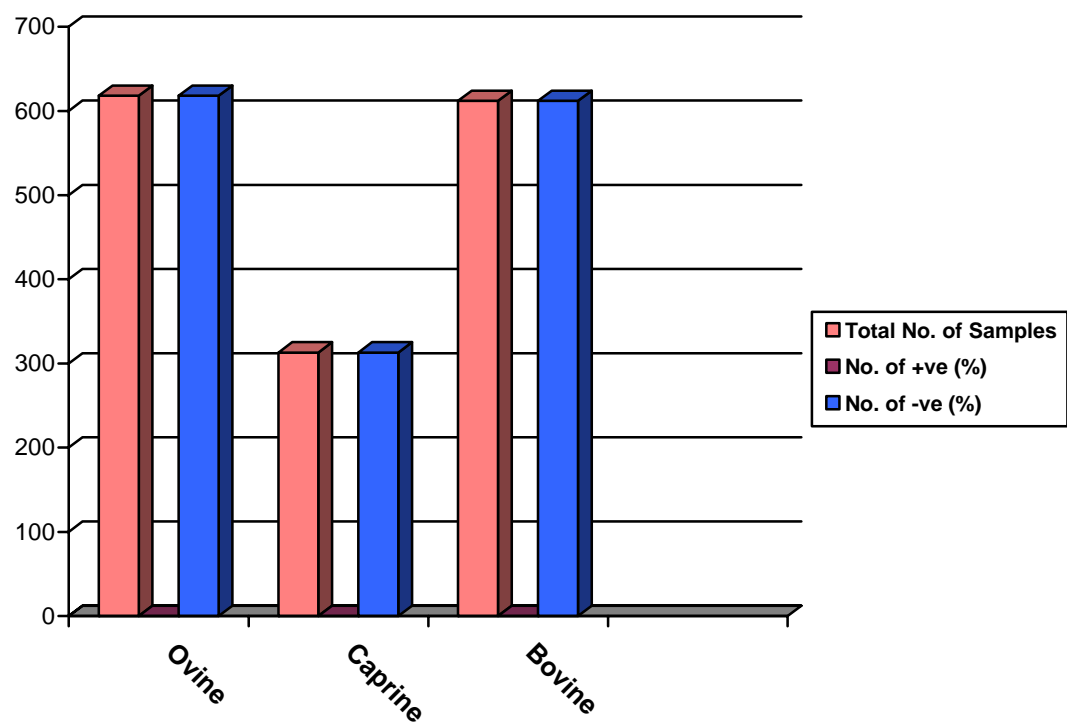
Table .2. Result of IgM ELISA during 2001

<b>Locality</b>	<b>Animal species</b>	<b>Total</b>	<b>No. of +ve (%)</b>
<i>River Nile</i>	Ovine	50	-
	Caprine	35	-
	Bovine	70	-
Kassala	Ovine	40	-
	Caprine	25	-
	Bovine	30	-
Red Sea	Ovine	49	-
	Caprine	60	-
	Bovine	57	-
S. Darfur	Ovine	86	-
	Caprine	38	-
	Bovine	70	-
W. Kordofan	Ovine	100	-
	Bovine	79	-
Khartoum	Ovine	40	-

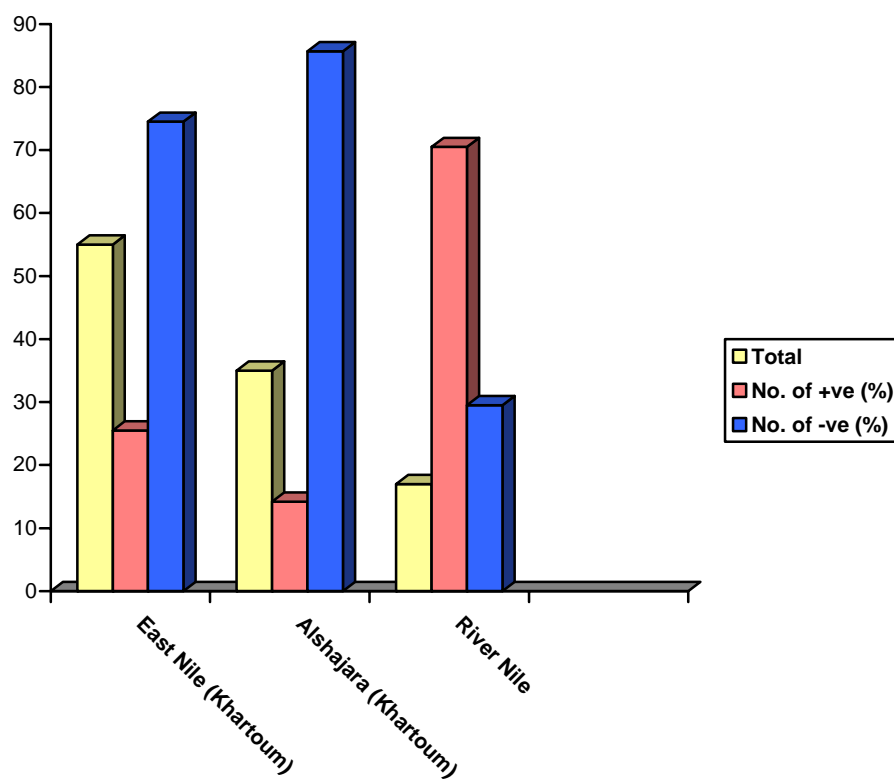
	Caprine	30	-
	Bovine	80	-
Omdurman	Ovine	45	-
	Bovine	30	-
E. Nile	Ovine	50	-
	Caprine	80	-
	Bovine	45	-
<i>White Nile</i>	Ovine	80	-
	Bovine	75	-
Blue Nile	Ovine	78	-
	Caprine	45	-
	Bovine	76	-

**Figure.8. Result of IgM ELISA 2001**

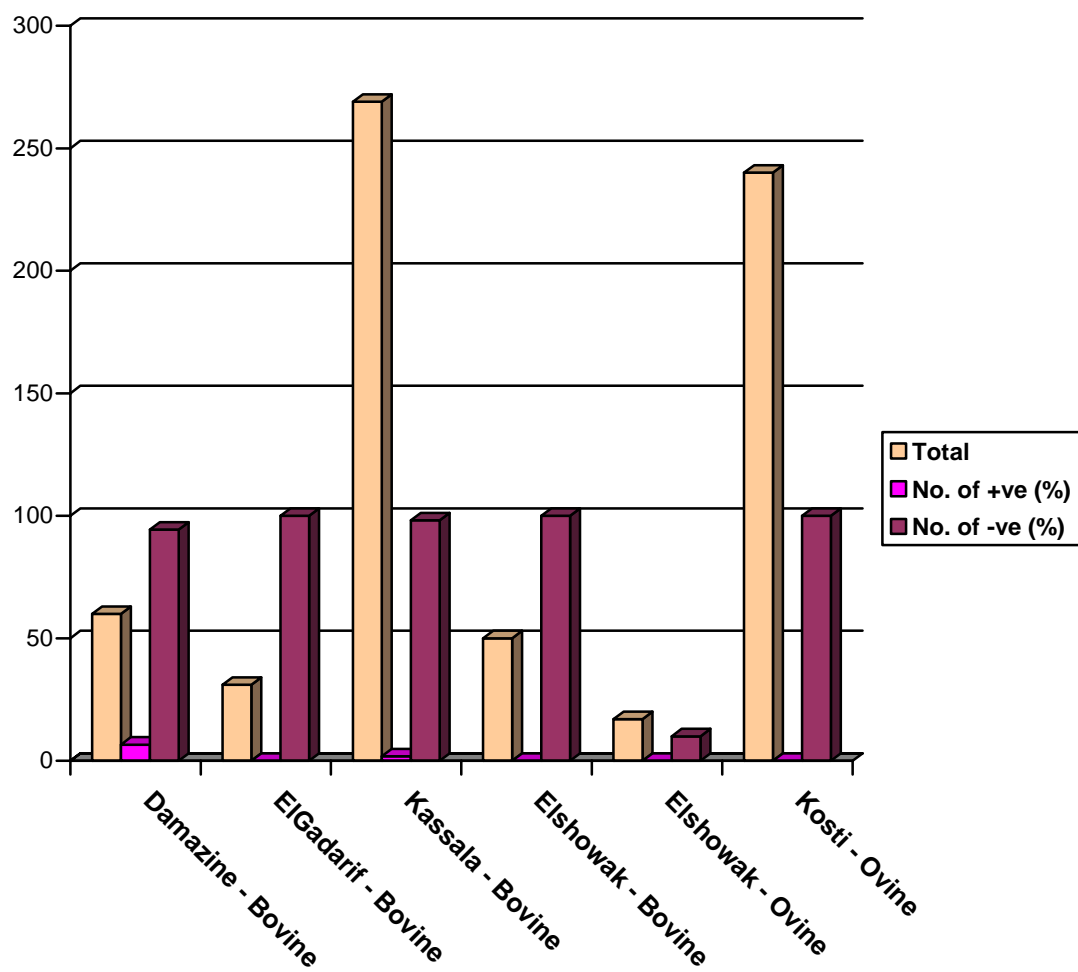




**Figure .9. Overall Results for IgM ELISA during 2001**



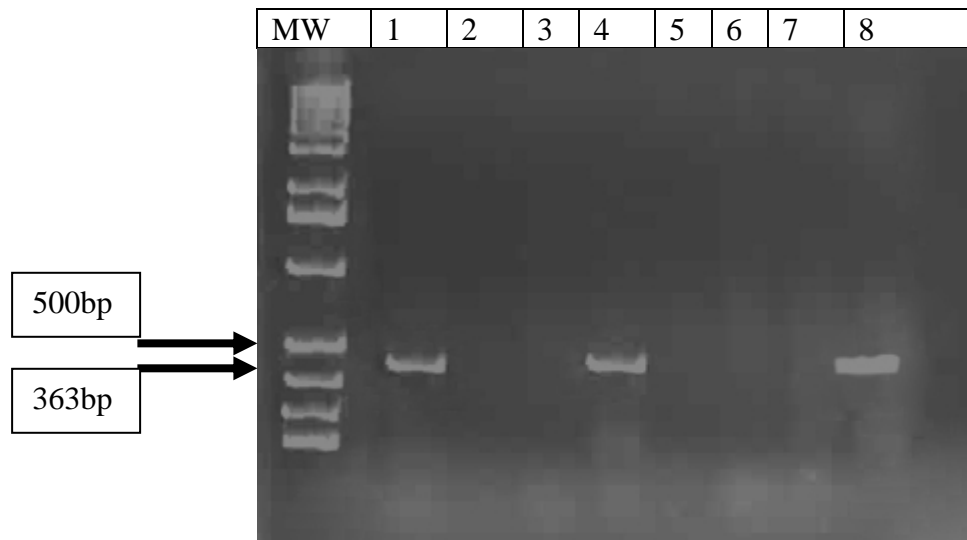
**Figure .10. Overall results for IgM ELISA during the year 2002**



**Figure.11. Overall results for IgM ELISA during the year 2005**







**Figure.12. Detection of 363 bp specific RVFV PCR Product using RT-PCR from clinical samples**

M= Molecular weight marker (100bp ladder)

1= RVFV Vaccine Strain (Smithburn) as positive control.

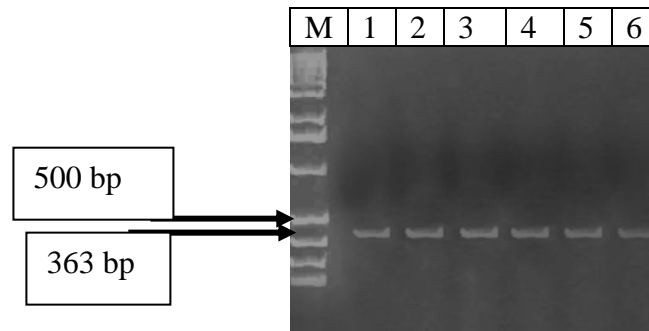
2=RNA extracted from non infected Vero cell negative control

3= RNA extracted from serum sample.

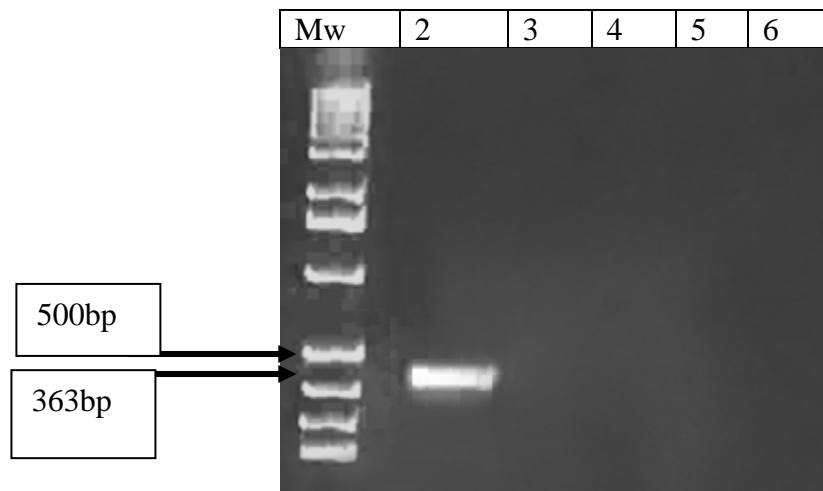
4= RNA extracted from spleen of aborted fetus

5, 6, 7 RNAs extracted from serum samples.

8= RNA extracted from liver of aborted fetus



**Figure. 13. Sensitivity of amplification of RVFV genomic RNA**  
M= Molecular weight marker (100bp ladder)  
1 -6 serial dilutions RVFV genomic RNA extracted from  
Vero cell infected with the vaccine strain (Smithburn)



**Figure.14. Specificity of RT-PCR for RVFV Genomic RNA extracted from cell culture Infected With the vaccine strain (Smithburn).**

Mw= Molecular weight marker (100bp ladder)

2= Rift Valley Fever Virus vaccine strain (Smithburn)

3= Epizootic hemorrhagic disease virus (EHDV)

4= Bluetongue Virus. (BTV)

5= Crimean Congo hemorrhagic fever virus. (CCHFV)

6= RNA extracted from non infected vero cell as control.

The 363 bp PCR amplification product was detected only with RVFV RNA.

## CHAPTER FOUR

### DISCUSSION

Rift Valley Fever (RVF) is an arboviral disease transmitted by mosquitoes; it affects primarily ruminants causing high mortality in offspring and abortions in pregnant females. The disease occasionally affects humans, with clinical picture that ranges from a mild febrile case to hemorrhagic fever with complications such as hepatitis and retinitis (Laughlin *et al.*, 1979).

Epidemics of RVF in Southern Africa occur in 5-10 years cycles, but in dry semiarid zones of Eastern Africa the periodicity is 15-30 years. During prolonged droughts RVF virus may entirely disappear from large areas due to death of reservoir drought-resistant mosquitoes (FAO, 1995).

The disease was previously reported in Sudan based on serological survey that indicated presence of antibodies to RVF virus in animals and human sera, (Findlay, 1936, Eisa *et al.*, 1977, 1980, 1984, Watts *et al.*, 1994, and Kambal, 1997) From all the data collected by aforementioned authors, it seems that RVF was prevalent in subclinical form, but the lack or poor of reporting system of the clinical cases did not reflect the real situation, because indigenous animals may not show clinical signs due to innate resistance, (FAO, 2002). The virus activity may be revealed by random isolations from mosquitoes or by occasional human disease, (FAO, 2002).

Many serological tests were used for diagnosis of RVF such as hemagglutination inhibition, complement fixation; agar gel immunodiffusion and neutralization test (Meegan *et al*, 1989). These methods were replaced by IgM antibody-capture or antigen-capture ELISA (Peters *et al.*, 1989). ELISA test has been developed with an inactivated viral antigen to provide safe and highly accurate alternate so it has the advantages over serum neutralization test which is specific and sensitive but time consuming and constitutes hazard to the laboratory diagnosticians as it requires the use of live virus (Swanepool and Coetzer, 1994) found that; ELISA was sensitive and specific in detecting RVF viral antibodies in comparison with plaque reduction neutralization test (PRNT). Similarly, Niklasson *et al.*, (1984) found a close correlation between ELISA and PRNT in detecting RVF viral antibodies in serum samples from human RVF vaccinees. Moreover, serum neutralization and PRNT were not recommended for use outside endemic countries unless a high level of biocontainment was available in laboratories. In addition the IgG and IgM ELISA were sensitive tests. There was no evidence of serological cross reactivity of RVF with other African phleboviruses which could obscure the diagnosis of RVF (Swanepool and Coetzer, 1994), also ELISA can detect earliest immunological responses to infection with RVFV and this is in agreement with Paweska *et al.*, (2003) who found the sensitivity of both ELISA (IgG and IgM) 99.05% to 100% and from 99.1% to 99.9% respectively. It was also stated that; detection of IgM by ELISA was a reliable marker of infection. Antibodies appear 5 to 14 days after onset and coincided with clinical improvement (Peter. 2005).The overall prevalence rate of IgG

antibody during the year 2001 was ranging from 3.4% to 10% in animals from Buram (Western Sudan) and Shandi (Northern Sudan) respectively. These foci (Shandi and Buram) of RVF may present a hazard when suitable environmental conditions prevail, when modifications in the ecology, biology of the virus and environmental conditions appear to be conducive to the emergence of the disease outbreaks, (Meegan and Bailey., 1989; Peters *et al.*, 1994). No IgM antibody was detected during 2001 in all sera tested.

The overall results obtained in this study were in contrast with that reported by Eisa, (1984) who stated widespread and high prevalence of RVFV antibodies in Sudanese livestock. This may be due to the drought and desertification that occurred in Sudan since mid 80s have led to the disappearance of the virus from most of the infected areas in Sudan and limited virus distribution to localized foci. The illegal deforestation in the rich savanna area may also lead to the disappearance of the reservoir hosts.

In July 2002 there was an unusually heavy rainfall and flooding that persisted for three months and this led to increase in vectors populations. In August 2002 a pyrexia disease spread among cattle in every locality in Khartoum and River Nile states manifested in high abortion rates reaching up to 70% and deaths of aborted feti. This raised the suspicions of RVF outbreak,

Following the spread of the disease a field survey was carried out to investigate and diagnose the condition, the overall prevalence rate of anti-RVFV IgM antibody was 28.9% among animals in the two states. This is quite similar to that reported by Eisa *et al* in 1977 and 1980 who

stated that a febrile illness spread among cattle in the Kuku dairy farm, calves were severely affected experiencing 40% mortality and 100% morbidity. This is in agreement with the fact that cattle usually show the highest prevalence of antibodies (65-95%) during RVF outbreaks (FAO, 2002).

In some of the sera tested during 2002, the optical density (OD) value of the negative control antigen well, was higher than for the positive antigen; this means that this serum sample may contain antigen i.e. the blood sample was taken during the acute phase of the disease (viraemic blood ). This was later proved using the direct ELISA for Ag detection.

Antigen detection by antigen-capture ELISA performed on serum or other samples (tissues of aborted feti) can be used to detect most hemorrhagic fever viruses, (Ksiazek, 1992; Towner, 2004).

In this study detection of RVFV antigen was tried by Ag capture ELISA using tissues (Liver, Spleen and brain) and serum samples, from aborted feti; tissues gave obvious color development while 3 serum samples were positive with lesser color intensity development.

IFA test has certain advantages as a serological technique, as it detects antibody response at an early stage and demonstrates antibody at high levels comparable to any other techniques. Moreover, antigen slides can be stored frozen, ready for immediate use; results can be obtained in about an hour. Sheep and cattle have a tendency to produce non-specific fluorescence of control cells at low dilutions (Swanepoel. *et al*, 1983). IFA test can also be used to detect viral antigen directly in impression smears of tissues of RVF-infected animals, particularly liver or fetal



brain ((Swanepoel *et al*, 1986). In this study IFA test was tried for the diagnosis of RVF antibodies. 23 out of 100 bovine serum samples show obvious fluorescence,

In comparison, ELISA and IFA tests were technically simpler than neutralization method because the latter requires high containment facilities. However, the reagents were relatively easy to obtain. IFA is very subjective, results may vary between observers. Since ELISA reagents are of high quality, it appears to be a precise and technically feasible method for detecting RVFV antibodies. The degree of agreement between the two tests (92%) is high enough to the IFA test to be used for the diagnosis of RVF infection.

Molecular techniques have also been applied for the detection of RVFV nucleic acid. Hybridization assay exhibited low sensitivity (Knauert *et al.*, 1989) and RT-PCR was successfully used to detect RVFV infected mosquitoes (Ibrahim *et al.*, 1997). In our study RT-PCR was used successfully to detect RVFV vaccine strain (Smithburn) propagated in Vero cells tissue culture and in tissues from aborted foeti. It was specific, sensitive, rapid, and easy to perform. RT-PCR product was not detected when RNA samples from BTV, EHDV and CCHF were applied to this RT-PCR. Also the single tube procedure reduced drastically the potential risk of contamination. Moreover, the detection limit of the RT-PCR appeared to be very low and might be useful for testing samples with very low viraemia, (Sall *et al.*, 2001). Despite some limitations in terms of its sensitivity, its cost and the level of expertise required of technicians, RT-PCR method is a rapid, sensitive, specific

and reliable assay for early detection of RVF infection even minimal amount of viral RNA is present,(Sall *et al.*, 2001).

It should be used routinely in conjunction with IgM ELISA for early diagnosis of suspected cases of RVF infection.

Mosquitoes remain one of the most important groups of insects which transmit a number of fatal diseases one of them is RVF.

Abugroon, *et al* (1988) stated that during mosquito Population Studies in the three towns of the capital of the Sudan, nine *culicine* species were encountered. *Cx. Quinquefasciatus* and *Cx. Univittatus* were most dominant and widely distributed species of the genus *Culex*. The study also showed a localized occurrence of *aedes egypti* and *aedes Caspui*. *Anopheles arabiensis* and *Anopheles pharoensis* were the only *anopheline* species found in the area.

Hussien (2002).stated that the most commonly used tool for collecting larvae were the dippers, which vary in size and shape but should always be white in color to facilitate collection of larvae. *anophelines*, *aedines* and some *culicines* were reported, the dominant species of *Anopheline* Mosquitoes was *An. Arbiensis*. Population density of *An. Arbiensis* was recorded during all months of the years except in May. Two peaks were observed, the major peak being the end of the rainy season (September, October) and the minor one at winter time (December, January). (Hussein, 2002).

A general survey was conducted in July 2005 which covered all Khartoum State, mosquitoes were collected from the following areas: Soba Gharb, Soba Sharg, Rimaila, AlShigailab, Jabra, Burri, AboSieid,

Arkawet, Al Gamair, Alban koko, IdBabikir, Dal Company project, AlGezeira, Dar AlSalaam, AlSarha and AlGaily.

The higher densities were reported in AlShigailab 11.6%, Soba Gharb 11%, Soba Sharq 99%, Burri 8 % and Arkweet 7%. The *Anopheles* species identified were: *Anopheles arabiensis* which is mainly breed in the domestic habitat. *Anopheles phrensis* collected mainly from farming areas. A total of 853 *Culex* larvae were collected from the above mentioned Stations mainly from contaminated water in farms and domestic habitat. *Aedes* mosquitoes have not been found in Khartoum so far. *Cx. Quinquefasciatus* had become most dominant because of its ability to exploit the range of breeding water available specially the highly polluted ones. It breeds in irrigated canals open drains septic tanks soakage pits small ditches, rain pools foot and hoof prints and metallic and other water containers. According to Emile (1986) the species can utilized all types of habitats created through urban growth. It was even more dominant than *An. Arabiensis* which has greater temperature endurance as reported by Omer and Cloudsley (1968). El Rayah and Nawal (1983).

*Cx. Quinquefasciatus* together with the other species newly reported in the area seems to have been introduced in the last decade as a result of urban growth and lack of control measures. *Cx. Antennatus* and *Cx. Pefuscus* which favours small grassy ditches are now spread with intensive agriculture. *An. Pharoensis* frequent in Gazera scheme might have reached Khartoum with the Managil branch which waters the green belt (12km from Khartoum).

*An. Arabiensis*, the main malaria vector in Central Sudan is reported in this survey mostly from suburban farming, seemed also to have adopted to Urban conditions by breeding in tannery water and complete shaded ditches. *An. Arabiensis* (*An. Gambia* species b) is the only member of *Gambia* complex present in the Sudan, Omer and Cloudsley Thomason (1968).

Khartoum city compared to Khartoum North and Omdrman had the richest breeding sites both in numbers and varieties because of greater urban practices utilizing water and the siphon drainage network which is badly managed. The cultivated landscape around the city which more or less provides fresh water for mosquito breeding is also very extensive. Under such a conducive situation there is a direct threat to public health from outbreak of unknown arboviral diseases. Beside *Cx. Quinquefasciatus* the well known vector of *filariasis*, *Cx. Univittatus* *Cx. antennatus* and *Cx. theieri* were incriminated in arboviruses transmission Ralph (1985).

*Wacheraia bancrofti* was found to adapt readily to *Cx. Quinquefasciatus* Wharton (1960). Tests in Kenya and Tanzania revealed that *Cx. Quinquefasciatus* acquirement to *Wacheraria* infections were 60-90%. Magayuka and white (1972). In crowded cities of eastern Africa coast *Cx. Quinquefasciatus* was evidently an important vector.

The ability of *Cx. Quinquefasciatus* to transmit arboviruses like chikungunya virus was reported from southern Tanzania Van Someren (1966) Woodall (1965).

The wide distribution of *Cx. Quinquefasciatus* coupled with influx of people from bancroftian filariasis endemic areas towards Khartoum, May favour the flare up of this disease.

All these mentioned data show a wide distribution of mosquito population in Khartoum state which constitutes a major public health hazard to the community.

## **Conclusions:**

In conclusions from all the data collected by previous authors, it seems that RVF in Sudan is prevalent in subclinical form, but the lack and poor of reporting system of the clinical cases did not reflect the real situation. In the year 2002 outbreaks of RVF were diagnosed in Khartoum and River Nile States by IgM ELISA and RT-PCR. The combined results of RT-PCR and IgM detection were in 100 % concordance with the results of virus isolation.

This limited investigation about mosquitoes showed that Khartoum State the capital city of Sudan and their suburban localities host high density of mosquitoes which established as a result of urban and agricultural increased utility of water coupled with poor sanitary awareness.

#### Recommendations:

Continuous monitoring of RVFV, in sentinel herds and in blood from human volunteers, through routine surveillance for virus and associated vector transmission would be the backbone of the epidemiologic studies on RVF in Sudan. In the absence of clinical hemorrhagic disease, sentinel cattle herds may provide an effective approach for monitoring animal health and incursion of RVFV isolates.

Further investigations are required to know the reservoir of RVFV, the situation in human and an entomological study is a must.

The application of Remote Sensing Satellite (RSS), Geographical Information System (GIS) and bioengineering to study the molecular epidemiology of the emerging viral infection should facilitate detection of an active RVFV infections, thus enhances humans and animal health monitoring. Vaccination and control programs for RVF could be facilitated by development and administration of a killed vaccine. Because of the segmented nature of the virus and the presence of different strains of RVFV, prevention of the disease by vaccination with attenuated live virus vaccines is a valid option. The attenuated vaccines should only be administered during the period of the year when the vectors are inactive. In

addition, the use of a recombinant vaccine may limit the level of transmission of RVFV and the extent of RVF in the near future.

Extensive sprays must be applied in the houses, farms every possible mosquito breeding sites, also the use of insects repellent and impregnated nets to minimize or completely destroy the mosquito population to break the life cycle of the virus,

Community awareness about the disease and the vector is the responsibility of all.

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## **Appendices**

### **Appendix 1**

#### **1. Deionized distilled Water (DDW):**

Water was distilled to get rid of salts and passed through deionizer to get rid of ions. DDW was used in the preparation of solutions and washing of equipments.

### **Appendix 2**

#### **2. Phosphate Buffer Saline (PBS)**

##### **Solution A**

NaCl.....	16 gm
KCl.....	0.4 gm
Na <sub>2</sub> HPO <sub>4</sub> (anhydrous).....	2.3 gm

KHPO<sub>4</sub>.....0.4 gm

DDW completed to.....1500ml

### **Solution B**

MgCl<sub>2</sub>(hydrous).....0.426 gm

DDW completed to.....200 ml

### **Solution C**

CaCl<sub>2</sub>.....0.264 gm

DDW completed to.....200 ml

The three solutions A, B and C were autoclaved separately at 121 for 30 minutes and left to cool. The solution A was added to solution B, and then followed by solution C, then the mixture was completed to 2000 ml with sterile DDW. The pH was adjusted by adding a few drops of neutral red.

## **Appendix 3**

### **3. Phosphate Diluent:**

NaCl .....16.0gm

KCl.....0.4gm

Na<sub>2</sub>HPO<sub>4</sub> (anhydrous)..... 2.3gm

KHPO<sub>4</sub>.....0.4gm

DDW completed to.....2000ml

The solution was sterilized by autoclaving at 1215C for 30 minutes.

## **Appendix 4**

### **4. Trypsin (7.5%)**

Trypsin powder.....37.5gm

PD completed to.....500 ml

The solutions were sterilized by filtration.

## **Appendix 5**

### **5. Tris buffer (tri-hydroxymethyl amino-methane)**

Tris..... 2.42gm

0.2M HCl.....76.7ml Adjust to pH7.4

DDW completed to.....400 ml

This is a 0.05M solution. The solution was sterilized by autoclaving at 115°C for 20 minutes. Stored at 4°C till use.

## **Appendix 6**

### **6. Mounting glycerol buffer:**

#### **Fluorescent antibody rinsing buffer(FA)**

Na<sub>2</sub>CO<sub>3</sub>.....11.4gm

NaCl..... 8.5gm

DDW completed to.....1000ml

Adjust pH 9.0-9.5, this is a 4X solution and should be diluted with

DDW to 1X. Keep in a tightly stopper container at room temperature.

Mounting glycerol buffer is prepared by mixing glycerol and FA buffer in equal amount.

## **Appendix 7**

### **6. Tris-boric acid- EDTA (TBE)**

Tri-hydroxymethylamino-methane 0.8M.....107.81g

Boric acid 0.89 M.....55.03gm

Ethylene-diamine-tetra-acetic acid (EDTA) 0.2 M 2H<sub>2</sub>O.....7.44gm

DDW completed to.....1000 ml

The mixture (10XTBE was dissolved on magnetic stirrer, kept at room temperature and then used as 1x concentration for gel preparation and electrophoresis.).

## **Appendix 8**

### **7. 1% agarose gel**

One gm of agarose powder (Molecular biology grade) was suspended in 100 ml of tris-boric acid EDTA (TBE) buffer. The suspension was heated in microwave oven for 3 minutes until the solution was clear. When cooled, 40 ml of the melted agarose was poured in the gel try loaded with a comb.